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Annual Report 1992



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NATIONAL INSTITUTE OF
~~DIABETES~~ AND DIGESTIVE
AND KIDNEY DISEASES

ANNUAL REPORTS

DIVISION OF INTRAMURAL
RESEARCH

October 1, 1991 to September 30, 1992

TABLE OF CONTENTS

PREFACE

Dr. Phillip Gorden, Director	I
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DIVISION OF INTRAMURAL RESEARCH

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PROJECT REPORTS

MATHEMATICAL RESEARCH BRANCH

Summary	1
Mathematical formulations and analysis relevant to experimental neurophysiology	9
Mathematical description of substrate transport in capillary-tissue structures	10
Mathematical description of cellular neuroelectric signal transmission	11
Electrical and chemical oscillations in coupled cell systems	12

LABORATORY OF CELLULAR AND DEVELOPMENT BIOLOGY

Summary	13
Protein nucleic acid interactions: chromatin structure and function	24
Study of ribonuclease and its inhibitor from bacillus amyloliquefaciens	25
Studies of folic acid (dihydrofolate reductase) and Vitamin A (Beta Carotene)	26
Synthesis and transport of lipoprotein and hepatic lipases in tissues and cells	27
Ultrastructural immunocytochemistry of lipid metabolism in cultured cells and tissues	28
Large-scale production and purification of compounds with biological activity	29
Regulation of developmental gene expression	30
Regulation of adipocyte metabolism	31
Control of gene expression in early mammalian development	32
Chromatin structure in regulation of mammalian gene expression	33
Mechanism of polyprotein processing in retroviruses	34

LABORATORY OF BIOCHEMISTRY AND METABOLISM

Summary	35
The role of the carbohydrate moiety of glycoprotein in cellular activity	44
Enzymatic basis of detoxication	45
Polysaccharides in morphogenesis	46
Thermodynamic and kinetic studies of protein structure and enzymic mechanisms	47
The role of the nuclear envelope in intracellular protein sorting	48
Tissue specific and hormone regulated gene expression	49
Inherited Disorders of Lysosomal Function	50
Electrochemical ion gradients as a mechanism of cellular message transmission	51
Cell regulation by Hormones, Growth Factors, Autoantibodies, and Oncogenes	52
Endocytosis, secretion and compartmentalization in mutant CHO cells	53
The role of intracellular traffic in HIV infection	54
Cell specific activity of elements within the HIV- LTR	55
Direct measurement of forces between membranes or macromolecules	56
Physics of ionic channels and other proteins with aqueous cavities	57
Structure and physical properties of DNA and DNA - protein complexes	58
Histamine release on a hydration of Granule matrices	59
Cell-cell fusion due to influenza hemagglutinin	60

LABORATORY OF CELL BIOLOGY AND GENETICS

Summary	61
Cytogenetic	75
Mechanisms of hormone and transmitter secretion	76
Vitamin C: Biochemistry, Molecular Biology and Human requirements	77

LABORATORY OF BIOCHEMICAL PHARMACOLOGY

Summary	78
Biochemistry of sulfur-containing compounds	90
Aldoheptose biosynthesis and its regulation and hepatitis non-A, non-B	91
Mammalian transposons	92
Bacteriophage T4 gene expression	93
Chemistry and Function of Microtubules	94
Structure and function of the tryptophan synthase multienzyme complex	95
Noncovalent: intermolecular interactions in biochemistry	96

Enzymatic mechanisms of DNA replication: The bacteriophage T4 system	97
Structure and interactions of biologically important macromolecules	98
Polyamine biosynthesis and function	99
Yeast RNA virology	100
Membranes Cytoskeleton and Secretion	101
Adenylate Cyclase and Other Extracellular Products of B. Pertussis.....	102

LABORATORY OF CHEMICAL BIOLOGY

Summary	103
The core loop interaction that controls protein folding	113
Studies of protein folding	114
Trans-acting factor(s) controlling globin gene expression	115
Sickle cell anemia: The intracellular polymerization of HbS	116
The mechanism of antigen-antibody interaction	117
The development of non-invasive methods to assess sickle cell patients	118
Regulation of beta globin gene expression	119
Laboratory model of adult globin gene expression	120
Trans-activating factors and globin gene expression: A direct approach	121
The Epsilon Gene Silencer: Characterization of Trans-acting Factor	122
Expression of Human Erythropoietin Receptor Gene in Transgenic Mice	123
Effect of hydroxyurea on fetal hemoglobin synthesis in Congenital Hemoglobin Disorders.....	124
Cytogenetic investigations of patients with genetically determined disorders	125
AIDS: Transcriptional Regulation by TAT-Protein and LTR of HIV in Vitro.....	126
Coordinated Expression of Human Beta Sickle Antilles and Human Alpha Globin in Transgenic Mice.....	127
Analysis of the Epsilon Globin gene flanking sequences	128
Trans-regulation of human globin genes	129
The erythropoietin receptor and genetic control red cell development	130
Mechanism(s) of enhanced gamma globin gene expression in patients	131
Production and Characterization of HIV TAT	132
Characterization of Epsilon-Globin silencer	133
Globin gene expression and the Treatment of Hemoglobinopathies	134
Globin Expression in an Erythroid Progenitor Culture System	135

The Development of Rheological Methods to Assess Sickle Cell Patients	136
Transcriptional Regulation of Human Erythropoietin-receptor Gene Expression	137
Covalent Modification of Hemoglobin in Hydroxyurea Treated Patients.....	138
Erythropoietin Receptor: Transcriptional Control.....	139

LABORATORY OF CHEMICAL PHYSICS

Summary	140
Molecular dynamics and vibrational characteristics of membrane assemblies	144
Asymmetric synthesis: Structure, stereochemistry, and NMR	145
The structure and dynamic properties of macromolecules	146
Structure and interaction of biomolecules	147
Electric and molecular structural investigation	148
Dynamics of proteins and studies on sickle cell disease	149
The physics and chemistry of photoreception	150
Macromolecular dynamics and assembly reactions	151
Spectroscopic investigation of membrane lipids and models	152
Theoretical studies on the dynamic aspects of macromolecular function	153
Nuclear magnetic resonance: New methods and molecular structure determination	154
Conformation and dynamics of biological macromolecules	155
Structural studies of AIDS proteins by NMR	156
Determination of three-dimensional structures of macromolecules in solution by NMR	157
Investigations of macromolecular structures and dynamics solution by NMR	158
NMR and other spectroscopic studies of molecular structure	159
Theoretical studies of dynamical processes in chemical physics and biophysics	160
Free Energy Conversion in Biology	161

LABORATORY OF BIOORGANIC CHEMISTRY

Summary	162
Pharmacologically active compounds from amphibians and other natural sources	192
Pharmacology and metabolism of biogenic amines and related compounds	193
Ion channels receptors and second messengers in the nervous system	194
Enzymatic oxidation of drugs to toxic and carcinogenic metabolites	195
Mechanistic enzymology of HIV proteins	196

Mass spectrometry of drugs, metabolites and natural products, proteins and oligonucleotides.....	197
Adenosine receptor agonists and antagonists	198
Interaction between second messengers	199
Analogues of Thyrotropin-releasing Hormone	200
Stereopopulation Control in Drug Delivery and Enzyme Simulation	201
Chemistry of imidazoles and bioimidazoles.....	202
Halogenated Biogenic Amines in Biochemistry and Pharmacology	203
Significance of Ligand Tautomerism in Biorecognition	204
Functionalized Congeners of Bioactive Compounds	205
Prosthetic Groups for Labeling of Functionalized Drugs and Peptides	206
Development of Drugs Acting at Adenosine Receptors	207
Bioindoles and oxindoles as medicinal and diagnostic agents.....	208
Novel Amino Acids for Conformational and Stereochemical Constraints in Peptides.....	209
Fluorinated Analogues of Bioactive Peptides	210
Chemistry and Biology of Novel Pyrimidine and Purine Nucleosides	211
Antimalarial Agents Based on Bioheterocycles	212
Development of multifunctional chemotherapeutic agents.....	213

LABORATORY OF MOLECULAR BIOLOGY

Summary	214
Studies of functions involved in genetic recombination	221
Studies of immunoglobulin gene rearrangement	222
Studies on mechanism of genetic recombination	223
Chromatin structure and function	224
Enzyme structure	225
Three-dimensional structure of cytokines, receptors and immune system proteins	226
Chemical and structural investigations of nucleic acids and related molecules	227
Nonheritable antibiotic resistance	228
Energy conversion in biology	229
Statistical thermodynamics of protein and polynucleotide systems	230
Thermal measurements of biomolecular systems	231
Influences of macromolecular crowding on biochemical systems	232
Developmental regulation of differential gene expression	233
Studies on the mechanism of retroviral DNA integration	234
AIDS related proteins: Structure and function	235

Control of gene expression during chicken erythrocyte development	236
Structural molecular biology	237
Channeling in the biosynthesis of histidine	238
Structural Studies of Molecular Recognition	239
Study of the Potential Use of Catalytic Antibodies Against AIDS	240
E.coli Genes Whose Expression is altered by salicyl alcohol	241

CLINICAL ENDOCRINOLOGY BRANCH

Summary	242
Thyroid Hormone Interactions with Cells and Proteins.....	243
Studies of Thyroid Diseases.....	244
Membranes and Secretion.....	245
Thyroid Hormone Secretion and the function of microtubules.....	246
Adenylate Cyclase and other extracellular products of B. Pertussis.....	247
Synthesis of Thyroxine transport proteins.....	248
Thyroid Hormone-Cell Interactions.....	249
Mapping of Triiodothyronine Responsive Genes.....	250
Regulation of Specific Rat Liver mRNAs by Thyroid Hormone.....	251
Molecular Biology of Thyroid Hormone Receptor.....	252
Effect of Thyroid Hormone on Synthesis of Myelin Basic Protein.....	253
Regulation of Anteroposterior Patterning in Early Frog Development.....	254
The Role of Xenopus-posterior (Xpo) in Early Frog Development.....	255

METABOLIC DISEASES BRANCH

Summary	256
Structure, secretion, and mechanism of action of parathyroid hormone	265
Studies on the mode of action of thyrocalcitonin	266
Study of hyperparathyroidism: Etiology, diagnosis, and treatment	267
Vitamin D resistance and related disorders	268
Regulation of mineral metabolism	269
Disorders of immune regulation in patients with systemic lupus erythematosus	270
Production and characterization of nephritic factor	271
Regulation of human immune response by complement	272
Immunosuppressive drug therapy in lupus glomerulonephritis	273

Renal biopsy pathology in systemic lupus erythematosus	274
Morphology of renal lesions in Pima Indians	275
Studies of Glomerular Cells Derived from Transgenic Mice....	276
Biology of insulin receptors in glomerular cells	277
Pathogenesis of murine lupus nephritis	278
Membranes lupus nephropathy	279
Glomerular lesions in mice transgenic for growth hormones	280
Role of IGF-I in biology of mouse glomerular cells	281
Biology of human mesangial cells	282
Idiopathic membranous nephropathy	283
Renal lesions in the ablation model.....	284
Identification of IGF-I binding proteins in mesangial cells in vitro.....	285
Interactions between transforming growth factor (TGF- β) and glomeruli	286
Glomerular lesions in non-obese diabetic mice	287
Advanced Glycosylation End Products, Effect on Mesangial Cells	288
Production of Metalloproteinases and TIMP1 by Glomerular Cells	289
Gene Expression in Microdissected Mouse Glomeruli	290
Effects of glucocorticoids on T cell activation	291
Transcriptional regulation of immunoglobulin genes.....	292
Studies of the Pathogenesis of Glomerulosclerosis.....	293
Degradation of Extracellular Matrix in Human Glomeruli.....	294

DIABETES BRANCH

Summary	295
Phosphorylation of the insulin and IGF-I receptor	301
Insulin gene expression and insulin action	302
Studies of insulin receptors in circulation cells in man	303
Antibodies to receptors.....	304
Positron emission tomography/NMR Spectroscopy	305
Acromegaly and growth hormone	306
Cellular hormone-like peptides	307
Morphologic studies of ligand binding to cells	308
Insulin receptors in syndromes of extreme insulin resistance	309
Biosynthetic labeling of the insulin receptor	310
Tyrosine-specific protein kinase activity associated with the insulin receptor	311
Use of SMS 201-995 in hormone secreting tumors	312
Transcriptional regulation of the insulin receptor gene	313
Mathematical modeling of glucose metabolism.....	314
Insulin receptor related receptor.....	315
Regulation of Gene Expression.....	316
Insulin-Cell Interaction.....	317
Insulin's Regulation of Glucose Transport.....	318

Alterations in Insulin's Action in Insulin-Dependent	
Diabetes Mellitus.....	319
Insulin's Regulation Hormone Binding.....	320
Counterregulation of Insulin's Action by Catecholamines.....	321
Alterations in Insulin's Action with Fasting/Refeeding.....	322
Glucose Transport in Mammalian Brain.....	323

CLINICAL HEMATOLOGY BRANCH

Summary	324
Study of immunology of blood cell deficiencies	329
Study of blood coagulation and diseases of hemorrhage and thrombosis	330

GENETICS AND BIOCHEMISTRY BRANCH

Summary	331
Gene expression and human genetics	336
Toxins and DNA repair in xenopus oocytes	337
Structure-function relationship of lysosomal enzymes	338
CD4 receptor structure/function project	339
Molecular studies of protein-DNA interactions	340
Thyroid Hormone Interactions with Cells and Proteins	341
Studies in thyroid diseases	342
Effect of thyroid hormone on synthesis of myelin basic proteins	343
Molecular Biology of Thyroid Hormone Receptor.....	344
Regulation of Anteroposterior Patterning in early frog development.....	345
Mapping of Triiodothyronine Responsive Genes.....	346
Regulation of Specific Rat Liver MRNAs by Thyroid Hormone.....	347

DIGESTIVE DISEASES BRANCH

Summary	348
Studies of Membrane Function.....	356
Cyclic Nucleotide Mediated Functions.....	357
Identification and characterization of Receptors for GI Peptides	358
Cellular basis of action of gastrointestinal peptides	359
Management of islet cell tumors	360
Receptors on gastric smooth muscle cells	361
Molecular characterization of receptors for GI peptides.....	362
Studies relating to the pathogenesis of hepatic encephalopathy and Fulminant Hepatic Failure.....	363
Immunologic studies of primary biliary cirrhosis	364
Studies of the natural history and treatment of chronic type B hepatitis	365
Studies of natural history and treatment of chronic hepatitis C.....	366
Trials of Therapies for Primary Biliary Cirrhosis.....	367

Immunological Studies in Chronic Viral Hepatitis.....	368
Studies of the Opiate System in Cholestatic Liver Disease.....	369
Studies of the Natural History and Treatment of Chronic Hepatitis B	370
Studies of the Natural History and Treatment of Chronic Hepatitis C	371

MOLECULAR, CELLULAR AND NUTRITIONAL ENDOCRINOLOGY BRANCH

Summary	372
Biosynthesis, glycosylation, and action of thyrotropin Clinical Trials of Recombinant TSH	378
Molecular biology of pituitary glycoprotein hormones and hypothalamic releasing hormones	379
Insulin-like growth factors	380
Insulin-cell interaction	381
Insulin's regulation of glucose transport	382
Alterations in insulin's action in insulin-dependent diabetes mellitus	383
Insulin's regulation of hormone binding	384
Alterations in insulin's action with fasting/refeeding	385
Mutations of the thyroid hormone receptor gene in patients with thyroid hormone resistance	386

LABORATORY OF MOLECULAR AND CELLULAR BIOLOGY

Summary	387
Function of DNA virus genomes in animal cells	391
Hormonal regulation of cell growth and differentiation	392
Studies on the metabolic defect in Sialuria	393
Regulation of HIV by AAV	394
Initial Intracellular Events of Steroid Hormone Action.....	395

LABORATORY OF ANALYTICAL CHEMISTRY

Summary	396
Analytical services and methodology	398
Initial intracellular events of steroid hormone action	399
The development of methods and materials for the study of medical problems	400
Professional practices of biomedical scientists	401
Interferon induction and action. The antiviral activity of nucleoside analogs	402
Physostigmine and analogs	403
Mammalian alkaloids	404
Structure-Activity relationships of colchicinoids based on tubulin binding	405
Analogues of nucleic acids and their components as potential anti-AIDS agents	406
Nortropane alkaloids	407

Analytical reagents from dihydrofluorescein	408
LABORATORY OF NEUROSCIENCE	
Summary	409
Receptors for neurotransmitters and drugs in brain and peripheral tissues	411
MOLECULAR PATHOPHYSIOLOGY BRANCH	
Summary	412
Molecular biologic studies on the cause of parathyroid neoplasia	418
Guanine nucleotide binding proteins as receptor- effector couplers	419
Studies on pseudohypoparathyroidism and related disorders	420
Studies on McCune-Albright Syndrome.....	421
Guanine nucleotide binding protein beta-gamma dimers: structure and function.....	422
Studies on nephrogenic diabetes insipidus.....	423
LABORATORY OF MEDICINAL CHEMISTRY	
Summary	424
Design and synthesis of drugs acting on central and peripheral tissues	452
Design, synthesis and evaluation of medicinal agents and research tools	453
Analogues of Nucleic Acids and Their Components as Potential Anti-AIDS Agent	454
Interferon Induction and Action. The Antiviral Activity of Nucleoside Analogs.....	455
Reactions and Immunochemistry of Carbohydrates.....	456
Evaluation of Potential Cocaine Antagonists.....	457
PHOENIX EPIDEMIOLOGY AND CLINICAL RESEARCH BRANCH	
Summary	458
Diabetes mellitus and other chronic diseases in the Gila River indian community	466
Complications and outcome of diabetic and prediabetic pregnancies	467
Gila River indian community autopsy and mortality study	468
Natural history of arthritis and rheumatism in the Gila River Indian Community	469
Cross-sectional and longitudinal study of "prediabetes" in the Pima Indians	470
Insulin resistance and the regulation of muscle glycogen synthase activity	471

Energy expenditures in Pima Indians: risk factors for body weight gain	472
WHO collaborating center for epidemiological and clinical investigations	473
Treatment of impaired glucose tolerance in Malmöhus County, Sweden	474
Genetics of non-insulin dependent diabetes mellitus	475
Regulation of skeletal muscle ribosomal protein S6 kinase by insulin	476
Contribution of protein tyrosine phosphatase to insulin resistance	477
Regulation of phosphatase by insulin	478
Epidemiology of complications of non-insulin-dependent diabetes	479
Kidney function in non-insulin-dependent diabetes mellitus	480
Insulin and hypertension in Pima Indians	481
Dietary survey of the Pima Indians of the Gila River Indian Community	482
Sodium - lithium countertransport and blood pressure	483
Insulin resistance in obesity and the association with lymph insulin kinetics	484
Regulation of gene expression by insulin	485
Insulin resistance in obesity and the associations with membrane phospholipid.....	486
Analysis of a Chromosome 4 Region Harboring a Gene controlling insulin action.....	487
Analysis of chromosome 19 in pima Indians	488
Mapping chromosome 11 in Pima Indians.....	489
Identification of Insulin Regulated Transcription Factors.....	490
SSCP Analysis of PP-1 Alpha and Gamma in Relationship to Insulin Resistance.....	491
Molecular Aspects of the Acute Insulin Response in Pima Indians	492

PROJECT NUMBERS

ACTIVE PROJECTS

Z01 DK 13001-19 MRB
Z01 DK 13002-20 MRB
Z01 DK 13004-19 MRB
Z01 DK 13020-03 MRB

Z01 DK 15100-22 LCDB
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TERMINATED PROJECTS

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Z01 DK 43233-03 MDB
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Z01 DK 45009-24 CEB
Z01 DK 45014-20 CEB
Z01 DK 45016-21 CEB
Z01 DK 45018-16 CEB
Z01 DK 45020-15 CEB
Z01 DK 45033-08 CEB
Z01 DK 45028-14 CEB
Z01 DK 45033-08 CEB
Z01 DK 45034-16 CEB
Z01 DK 45038-04 CEB
Z01 DK 45040-03 CEB
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Z01 DK 69043-03 PECR

Z01 DK 69044-02 PECR

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Z01 DK 69046-01 PECR

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Z01 DK 69050-01 PECR

INACTIVE PROJECTS

Z01 DK 24942-16 LBP

Z01 DK 25028-14 LCB

Z01 DK 43228-05 MD

Z01 DK 47014-21 DB

Z01 DK 48001-01 DB

Z01 DK 48004-01 DB

Z01 DK 48006-01 DB

Annual Report of the

Mathematical Research Branch

National Institute of Diabetes and Digestive and Kidney Diseases

Current research projects of the Mathematical Research Branch reflect a broad range of interests in the development and application of theoretical models and of quantitative methodologies for understanding biological systems.

This research involves several different collaborations within the Branch and with other research groups, both at the NIH and elsewhere. This report describes recent work in the areas of cellular and network neurobiology, electrical activity of secretory cells, microcirculation, renal physiology, cell energetics, and mathematical/numerical methodologies.

During the past year, international collaborative projects have involved foreign investigators at Hebrew University, Jerusalem (Department of Neurobiology), at the University of Oxford (Physiological Laboratory), and at the Australian National University. Invited presentations were given at distinguished symposia by J Rinzel (Annual Meeting, Society for Neuroscience, New Orleans; Annual Meeting, Society for Mathematical Biology, Berkeley; Annual Meeting for Computational Neuroscience, San Francisco) and by A Sherman (World Congress on Nonlinear Mathematics, Tampa). An invited paper was completed by W Rall (and coauthors) for an issue of Physiological Reviews to commemorate the land-mark publications of Hodgkin, Huxley and Katz in 1952. A research workshop entitled "Implications of dendritic models for neural network properties" was organized by W Rall and conducted at the Santa Fe Institute, Santa Fe, NM, Oct, 1991. J Rinzel was involved in organizing the six-week Summer Program in Mathematical Physiology (July-Aug, 1991 at the Mathematical Sciences Research Institute, Berkeley); several MRB staff were in invited participants. J Rinzel was invited to teach in the course, Methods in Computational Neuroscience, Woods Hole, MA and in the International School of Neural Modeling and Neural Networks, Capri, Italy. J Rinzel received the USPHS Outstanding Service Award.

Network Neurobiology.

The lamprey central pattern generator (CPG) for swimming. The isolated spinal cord of the lamprey can produce a traveling wave of neural activity as evidenced by repetitive bursts of action potentials recorded at the motor roots. The time delays between bursts measured at different segments and the burst periods typically fluctuate about some mean. The lamprey CPG has been modelled as a chain of coupled oscillators. We have added noise to the oscillator frequencies in this model and have developed a maximum-likelihood estimation of the coupling strengths. We are presently applying this maximum-likelihood estimation to experimental data. (T Kiemel and A Cohen, N Mellon: Univ MD)

Coupled oscillators near saddle loop bifurcations. For a class of neuron models, oscillations terminate as a parameter is varied due to a saddle loop bifurcation in which the period of the oscillation goes to infinity. We are studying two identical gap-junction coupled cells which are each near a saddle loop bifurcation. We have shown that generically the in-phase solution is unstable. We are presently investigating the stability of the anti-phase solution. (T Kiemel)

Z01 DK 55012-08 MCEB
Z01 DK 55013-06 MCEB
Z01 DK 55014-07 MCEB
Z01 DK 57000-26 LSB
Z01 DK 58002-01 LAC
Z01 DK 58005-17 LAC
Z01 DK 58010-06 LAC
Z01 DK 58014-03 LAC
Z01 DK 58017-02 LAC
Z01 DK 58018-02 LAC
Z01 DK 59601-05 LMC
Z01 DK 69029-04 PECR
Z01 DK 69038-03 PECR

Activity in slow synapse networks. In some neurons chemical synapse kinetics are much slower than spike generating processes. Specific kinetic schemes are transformed so that an averaging method based on differing time scales can be used to understand the dynamics of coupled neurons, for example bistability and bursting. The hysteresis which underlies this type of bursting can be generated from either a subcritical Hopf bifurcation in an isolated cell, or through network feedback effects. Both cases are analyzed. It appears that low order chaos is associated with such bursting. In the non-bursting, bistable situation, we use higher order approximations to describe the phase relationships of two oscillating cells; we find that the effects of slow coupling are fundamentally different than those of weak coupling. The case of nearly identical oscillators with nearly identical coupling has been thoroughly analyzed. Behaviors such as 6:7 phase entrainment, phase oscillations, in-phase stable, out-of-phase stable, and phase-walkthrough are predicted with the averaging theory; these have been observed for a particular numerical example. (P Frankel, J Rinzel and T Kiemel)

Oscillations of a thalamic network model. Previously we developed a model which exhibits postinhibitory rebound excitation, like the low-threshold spike (LTS) of thalamic neurons (with M Rogawski, NINDS). In order to understand the 10 Hz synchronized bursting oscillations in the mammalian thalamocortical system during deep sleep, we consider such model cells coupled by inhibitory synapses. When the synaptic current depends instantaneously on presynaptic voltage, two cells oscillate out-of-phase. However, when the synaptic current has a slow decay property, perfect in-phase synchrony can be realized. We have determined numerically the ranges for decay rate and synaptic reversal potential which yield these behaviors. A network of ten cells, coupled all-to-all, can also synchronize with slowly decaying synaptic currents. Moreover, with sufficient synaptic strength, the network may have several different stable activity patterns: with some fraction of the cells inhibited and the remainder oscillating in-phase. We suggest that the slow decay of GABA-b mediated inhibition is important for the hypothesized role as pacemaker of the thalamic reticular nucleus. (X J Wang and J Rinzel)

Cellular Neurobiology.

A-current modulation of thalamic neuron bursting. We constructed a semi-quantitative model for bursting which consists of rapid action potentials (Na spikes) which ride on the slow depolarized phase of the low-threshold spike (LTS). A T-type Ca current underlies the LTS. Upon release from hyperpolarization, the LTS is slowly terminated by the inactivation of this Ca current. A transient potassium current (A-current) is also de-inactivated during hyperpolarization. We update the model by including an A-current, and analyze its imposition on the T-current. The full model leads to LTS bursts with diminished Na spiking, suggesting that an additional inward current may contribute to the greater spiking as seen experimentally. Furthermore, the A-current can delay the onset of the LTS; this could have consequences in the coupling behavior of thalamic neurons. (M Rush and J Rinzel)

Models of hippocampal neurons.

We have been analyzing a detailed multicompartment model of hippocampal neurons. One aim is to reduce this system of over 100 variables to a simpler one which has qualitatively similar behavior. To this end we have developed several 2 compartment

models which show the same transitions from repetitive bursting to repetitive spiking as applied current is increased as does the full model. We are currently trying to understand mathematically the nature of this transition from bursting to spiking. A one dimensional map that approximates the dynamics of the 2 compartment model has been developed and may prove useful in this regard. The models we have developed have one or more slow variables. These different time scales have been utilized to develop asymptotic results. We have proved a series of theorems dealing with general systems of equations with slow variables. These can be used to reinforce numerical findings in the models. (P Pinsky and J Rinzel).

Dendritic neuron modeling. An invited review, "Matching neuron model complexity to experimental data", has been prepared for a special issue of Physiological Reviews to commemorate the land-mark publications of Hodgkin, Huxley and Katz in 1952. The review also includes new results. Its focus is on mathematical models whose increasing geometric complexity was designed to more nearly match the wealth of anatomical data that has recently become available for several neuron types. Complications include a significant shunt conductance (across the soma membrane) that is probably introduced by sharp microelectrodes, and nonuniformity of soma and dendritic membrane, including some discussion of nonlinear membrane properties. The review compares the results and implications of the more complex models with those of the earlier idealized models. Among the consequences are higher values estimated for membrane resistivity and the membrane time constant, with lower values for the dimensionless electrotonic length of dendritic trees; this enhances the theoretical efficacy of dendritic synapses. Many of the points made only briefly in the Review are documented in detail in three papers completed this year by Rall, Holmes, and Segev. (W Rall, RE Burke:NINDS, WR Holmes: Ohio U, JJB Jack: Oxford, SJ Redman: Australian Nat U, I Segev: Hebrew U and MRB,NIDDK)

A research workshop entitled "Implications of dendritic models for neural network properties" was organized by W Rall and conducted at the Santa Fe Institute, Santa Fe, NM, Oct.10-14, 1991 (sponsored by SFI). Twelve participants provided many explicit examples of important and interesting properties of neurons (involving nonlinear membrane properties and/or nonuniform distributions of synaptic inputs to dendritic membrane); such properties are completely neglected by the binary units assumed by most network modelers. Collaborations were identified that could offer special promise of demonstrating how network properties can be enriched by including realistic neuron properties in network models. A number of participants favored neuron models with an intermediate level of complexity. (W Rall, with workshop participants).

Interpretation of synaptic current measurements. We have carried out simulations in parallel with experimental measurements of calcium current in calyx nerve terminals of the chick ciliary ganglion. The calyx is large in area (it may cover half of the post-synaptic cell body) and very thin, implying that it may be electrically large and not well space-clamped. We sought to test the hypothesis that poor space-clamp could account for the observed skewing of I-V relations to positive voltages and failure of the current to decline towards the calcium reversal potential. Modeling the calyx as a disk, we confirmed that the ideal I-V relation is reduced in amplitude and shifted to the right when the thickness of the disk is small. With sufficient right shift, the curve may be monotonic in the experimental range of voltages. It was also apparent that under conditions of poor

space clamp, unclamped spikes can occur making it impossible to measure I-V relations, unless significant leak current is present after applying K-channel blockers. (A Sherman and E Stanley: BL, NINDS)

Electrical Activity of Insulin-secreting Cells.

Effects of electrical coupling among pancreatic beta-cells. It appears experimentally that bursting oscillations occur only in large ensembles of cells, such as their natural setting, the islet of Langerhans. We have previously reported that gap-junctional coupling can allow ensembles to burst, even when the individual cells are prevented from bursting by excessive channel noise. Because more recent evidence argues against channel noise as a major factor, we have proposed a new hypothesis that cell heterogeneity is responsible for the inability of isolated cells to burst. To test this hypothesis we have simulated clusters of cells with experimentally determined variances of parameters and coupling strength. We find that even a 10% variation from a set of parameters known to give bursting in a model cell yields a population in which few cells are capable of bursting in isolation. When coupled, however, synchronized bursting is observed. As found previously in clusters of identical cells, the spikes are out-of-phase and reduced in amplitude, leading to increased burst period. Because of deterministic coupling effects, the spike pattern is irregular, resembling experimental spikes and rather unlike those in isolated model cells. Thus, we learn that one should not expect single-cell models to replicate experimental courses of cells subject to coupling influences. (P Smolen, J Rinzel and A Sherman)

We are studying in detail coupling of pairs of cells using simplified beta-cell models. By using a combination of analytical and numerical techniques we examine the underlying basis for the coupling effects observed in large-scale, physiologically detailed simulations, as above. Using perturbation methods we derive reduced equations in a parameter regime where coupling is weak and stability of oscillations is also weak. Numerical comparisons reveal that the reduced equations give results in excellent agreement with the original system, but are much easier to study. We seek to isolate the physical parameters that are responsible for out-of-phase oscillations and also to extend the analysis to more than two oscillators. (A Sherman and HR Zhu)

Ionic channel mechanisms for beta-cell bursting oscillations. It is still not clear what slow change in ionic conductance underlies these oscillations, although slow voltage-gated inactivation of a calcium current and slow modulation of a potassium conductance are strong possibilities. We are collaborating with experimentalists to examine the importance of these possibilities. This effort involves modeling and concurrent refinement of new experimental protocols. As part of these efforts, we are beginning to examine the importance for bursting of a T-type calcium conductance recently found in rat and human beta-cells. (P Smolen and L Satin: Virg Comm Univ Med School and E Rojas: LCBG, NIDDK)

Renal Physiology.

Ammonium and bicarbonate transport along long-loop thin descending limb. Luminal fluid from proximal convoluted tubule of a juxtamedullary nephron is alkalized as it passes through the long descending loop of Henle (LDL). Three potential mechanisms of alkalization are: concentration of bicarbonate by water abstraction, direct bicarbonate entry, and NH_3 entry. We have used a mathematical

model of LDL to investigate these mechanisms. We used permeabilities of HCO_3^- , NH_3 , and NH_4^+ measured (in experiments designed with the help of our model) for subsegments of the chinchilla LDL, the osmotic water permeability of each segment, and appropriate parameters from the literature, including interstitial profiles obtained from in-vivo measurements, to calculate pH, $[\text{HCO}_3^-]$, and $[\text{NH}_3]$ of the luminal fluid as it descends the LDL. Our studies indicate that, while all three mechanisms individually contribute to LDL alkalization, NH_3 likely plays the dominant role. Our experiments showed that the outer medullary segment was the most permeable to ammonium, while the deep inner medullary segment was the most permeable to bicarbonate. (R Mejia, and M Flessner M Knepper:NHLBI)

Role of the kidney in acid/base balance. We have continued the development of a multinephron model for acid/base balance in the whole kidney, as experiments to measure the necessary parameters continued. A numerical algorithm that uses domain decomposition with parameter continuation to solve a system of differential-algebraic equations with 4th order accuracy in the nephrons has been enhanced to permit a variable step size larger than 2% of axial length, which allows computation of species concentrations with an accuracy exceeding 0.1%. (R Mejia and M Knepper:NHLBI)

Microcirculation.

Vasomotion. An integrated formulation for the vasomotion of small arteries has been completed. It incorporates the calcium and potassium ion transports, the membrane electrical activity and the calcium generated muscle contraction of the vessel wall's smooth muscle cells into the mechanics of an internally pressurized thick wall vessel cylinder. A more detailed description of the smooth muscle biochemistry, contraction velocity and stress development has been incorporated. The calcium entry results in the phosphorylation of the myosin light chain which determines the crossbridge cycling, the stress development and the contraction velocity. Our computed results reproduce all the features found experimentally in an excised isolated middle cerebral artery subjected to different intraluminal pressures for both conditions: intact and abolished myogenic response. (JM Gonzalez-Fernandez and B Ermentrout: U Pittsburgh)

Vasomotion in arteriolar networks. The network model consists of one main branch, terminal artery with a given intraluminal pressure, that gives rise to several branches in parallel, arterioles. Each arteriole possesses vasomotion capabilities, dependent on the local transmural pressure. There is no humoral or electrical connections between the arterioles. The vasomotion in one arteriole influences, through changes in its hydraulic flow resistance, the intraluminal pressure of the other arterioles. This, in turn, influences their oscillatory activity. As a result, for most of the cases studied, the arteriolar network oscillatory activity becomes synchronized. The synchronization attains more promptly when the proximal arteriolar resistance is larger; this could be relevant for the synchronization observed in sickle cell anemia. In some cases the model shows that no synchronization attains, but that the basic oscillatory behavior is replaced by a non periodic vasomotor activity. This is reminiscent of the experimentally observed non-periodic, non-synchronized behavior between neighboring arterioles, which has been assumed to be unrelated arteriolar behaviors. (JM Gonzalez-Fernandez and B Ermentrout: U Pittsburgh)

Selected Other Topics.

Cell energetics. We have previously described reaction-diffusion models for ATP and its byproducts that are based on data obtained using MDCK cells grown in monolayers. A model with cylindrical geometry, radial symmetry and allowing a variable distribution of mitochondria for oxidative phosphorylation has been developed and is being used to infer cell properties. The model predicts an inverse log-linear relationship between maximal [ADP] and species diffusivity. In addition, maximal [ADP] is about 20 times the average over the cell, both at rest as well as under stressed conditions. (R Mejia and R Lynch:U Arizona)

Solving reaction-diffusion equations by Monte Carlo methods. We have developed a numerical method based on random walks for solving equations in two or more space dimensions. Diffusion is simulated by Brownian motion of particles, and reaction is simulated by a process with an evolutionary flavor: Particles are selected for proliferation or extinction such that collectively they will represent, on average, the solution of the partial differential equation. We are exploring how to make this algorithm efficient enough to compete with standard methods. The method is of general interest because of its close connections with algorithms for the motion of vortices in fluids. (A Sherman and M Mascagni)

Journal Articles

- @120Cohen AH, Kiemel T. Intersegmental coordination: lessons from modeling systems of coupled non-linear oscillators, *Amer Zool* (in press).
- @120Holmes WR, Rall W. Electrotonic models of neuronal dendrites and single neuron computation. In: McKenna T, Davis J, Zornetzer SF eds. *Single Neuron Computation*. Boston: Academic Press, 1992;7-25.
- @120Holmes WR, Segev I, Rall W. Interpretation of time constant and electrotonic length estimates of multi-cylinder or branched neuronal structures, *J Neurophysiol* (in press).
- @120Holmes WR, Rall W. Electrotonic length estimates in neurons with dendritic tapering or somatic shunt, *J Neurophysiol* (in press).
- @120Mejia R. Solution of differential-algebraic equations for renal acid-base balance. In: Keyes D, Chan T, Meurant G, Scroggs J, Voigt R eds *Fifth Symposium on Domain Decomposition Methods for Partial Differential Equations*. Philadelphia: SIAM Press (in press).
- @122Rall W, Burke RE, Holmes WR, Jack JJB, Redman SJ, Segev I. Matching neuron model complexity to experimental data, *Physiol Rev* (in press).
- @122Rall W. Functional insights about synaptic inputs to dendrites. In: Eeckman Fed. *Analysis and modeling of neural systems*. Boston: Kluwer Academic Publishers, 1992:63-8.
- @122Rall W. Path to biophysical insights about dendrites and synaptic function. In: Samson FE, Adelman G eds. *The Neurosciences: Paths of Discovery II*. Boston: Birkhauser (in press).
- @121Rinzel J, Frankel P. Activity patterns of a slow synapse network predicted by explicitly averaging spike dynamics, *Neural Computation* (in press).
- @121Rinzel J, Sherman A, Stokes CL. Channels, coupling, and synchronized rhythmic bursting activity. In: Eeckman Fed. *Analysis and modeling of neural systems*. Boston: Kluwer Academic Publishers, 1992;29-46.
- @122Sherman A, Rinzel J. Rhythmogenic effects of weak electrotonic coupling in neuronal models, *Proc Natl Acad Sci* 1992;89:2471-4.
- @122Smolen P, Keizer J. Slow voltage-inactivation of calcium channels and bursting in the mouse pancreatic beta-cell, *J Memb Biol* (in press).
- @122Terman DH. The transition from bursting to continuous spiking in excitable membrane models, *J Nonlinear Science* (in press).
- @120von Kienlin M, Mejia R. Spectral localization with optimal pointspread function, *J Magn Reson* 1991;94:268-87.

@122Wang XJ, Rinzel J, Rogawski ML. Low threshold spikes and rhythmic oscillations in thalamic neurons. In: Eeckman Fed. Analysis and modeling of neural systems. Boston: Kluwer Academic Publishers, 1992;85-92.

@122Wang XJ, Rinzel J, Rogawski ML. A model of the T-type calcium current and the low-threshold spike in thalamic neurons, J Neurophysiol 1991;66:639-50.

@122Wang XJ, Rinzel J. Alternating and synchronous rhythms in reciprocally inhibitory model neurons, Neural Computation 1992;4:84-97.

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Mathematical formulations and analysis relevant to experimental neurophysiology.

PRINCIPAL INVESTIGATOR (Last either professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W. Rall Senior Research Physicist MRB, NIDDK

Other: I. Segev Fogarty Visiting Scientist MRB, NIDDK

COOPERATING UNITS (if any)

Dept. of Neuroscience, Hebrew Univ. of Jerusalem

LAB/BRANCH

Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.6

PROFESSIONAL:

1.3

OTHER:

.3

CHECK APPROPRIATE BOXES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

RESEARCH AREA. basic neuroscience involving structure/function relations for neuronal dendritic branching, dendritic spines, and synapses (also neuron populations, with cortical symmetry), and for such functions as synaptic transmission, amplification and dendro-dendritic interactions in the context of spatio-temporal input patterns, logical processing of input, and neural plasticity, as in conditioning and learning.

RATIONALE. Combine experimental data from neuroanatomy and from electrophysiology with biophysical models of nerve membrane (passive, synaptic and excitable) into a comprehensive theory which can lead to new insights and to testable theoretical predictions (leading to the design of better experiments). To do this we must create, explore and test mathematical and computational models with different degrees of complexity.

METHODOLOGY. Our methods include both analytical solutions and computational solutions of boundary value problems (for partial differential equations) in the tradition of classical physics. They include also the formulation and solutions of problems in terms of systems or ordinary differential equations; when this is done explicitly for a compartmental model of a neuron, it is possible to accommodate a remarkable variety of dendritic branching patterns and non-uniform distributions of membrane properties and of synaptic inputs.

RESULTS. For book chapters giving results, perspective, and references, see: "Single Neuron Computation" (T. McKenna, J. Davis & S.F. Zornetzer) Academic Press, 1992; "Computational Neuroscience" (E.L.Schwartz, ed.) MIT Press, 1990; "The Segmental Motor System" (M.D.Binder & L.M.Mendell, eds.) Oxford Press, 1990; "Methods in Neural Modeling" (C.Koch & I.Segev, eds.) MIT Press, 1989.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-13,002-20 MRB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Mathematical description of substrate transport to capillary-tissue structures.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. M. Gonzale-Fernandez

Research Mathematician

MRB, NIDDK

COOPERATING UNITS (if any)

Dept. of Mathematics
Univ. of Pittsburg

LAB/BRANCH

Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.33

PROFESSIONAL:

1.3

OTHER:

.03

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

This goal of this work is to develop mathematical models of the blood flow and transcapillary exchanges in capillary networks. An effort is being made to incorporate in the models of the histological structure of capillary networks as well as different flow patterns from available experimental information. In this model the extraction substrates with different chemical kinetics at the tissue site will be described. It is expected that this could be used in experimental situations where the extraction or different substrates are measured simultaneously, thus helping to infer the flow pattern features of the microcirculation. A model of the diffusion-consumption of oxygen in striated muscle containing myoglobin (facilitated diffusion) is being developed and pertinent numerical results examined. A description of the origin and control of vasomotion is being formulated, and applied to the study of microcirculation networks.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 34004-20 MRB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical description of cellular neuroelectric signal transmission.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Rinzel, Chief, MRB MRB, NIDDK

Others: A. S. Sherman Senior Staff Fellow P. Frankel pre-doc IRTA
P. D. Smolen NRC Fellow M. Rush pre-doc IRTA
Y. X. Li Fogarty Fellow P. Pinsky pre-doc IRTA

All in MRB, NIDDK

Cooperating Units: Lab Cell Biol & Genetics, NIDDK
Dept Pharmacol, VA Com Med Sch
Dept Chem, U California, Davis

Dept Mathematics, Ohio State University
Dept Mathematics, University of Chicago

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Mathematical Research Branch

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

4.5

PROFESSIONAL:

4.0

OTHER:

0.5

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

This project continues to focus on the formulation, analysis, and biophysical interpretation of mathematical models which describe various aspects of electrical activity of neurons and other cell types. Among the topics of current interest are: (i) integration of synaptic input delivered to the soma and dendritic branches of a neuron; (ii) propagation of action potentials along axons; (iii) stimulus-response and threshold properties for repetitive-firing of action potentials; (iv) complex bursting patterns of membrane potential oscillations which arise through endogenous membrane properties and/or intercellular coupling.

Because qualitatively related mathematical or biophysical problems may arise in other context, e.g. chemical and biochemical oscillations, or e.g. excitation-secretion coupling, this project may consider models from such applications.

Mathematical models of these phenomena involve systems of linear and nonlinear ordinary differential equations and parabolic partial differential equations. Solutions and their mathematical stability are determined by analytical and numerical methods drawn from both classical and modern applied mathematics. These methods may include finite difference or finite element numerical integration, theory. One goal of this project is to expose the qualitative mathematical structure for classes of models by exploiting simple, yet physiologically reasonable, equations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-PK-13,020-03 MRB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Electrical and Chemical Oscillations in Coupled Cell Systems

PRINCIPAL INVESTIGATOR (List either professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Sherman Senior Staff Fellow MRB, NIDDK

Other: J. Rinzel, Chief, MRB, NIDDK
M. Mascagni, Guest Worker, MRB, NIDDK
H.-R. Zhu Fogarty Fellow, MRB, NIDDK
Elis Stanley, BL, NINDS

COOPERATING UNITS (if any)

Biophysics Lab, NINDS

LAB/BRANCH

Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

.1

OTHER:

2.1

CHECK APPROPRIATE BOXES

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

We use mathematical models to study the mechanisms of oscillatory electrical activity arising from ion channels in cell membranes and modulated by intracellular chemical processes. We are interested in both the behavior of single cells and the ways in which cells communicate and modify each other's behavior.

Our main application has been to the biophysical basis of insulin secretion in pancreatic beta-cells. We have examined bursting oscillations in membrane potential and the role of electrical coupling between cells in the islet of Langerhans. Long term goals are to understand how the membrane dynamics interact with intracellular events to regulate secretion and to generalize to other secretory cells and neurons.

Our primary tool is the numerical solution of ordinary and partial differential equations. We use analytical, geometrical, graphical, and numerical techniques from the mathematical theory of dynamical systems to help construct and interpret the models. Perturbation techniques are used to get analytical results in special cases.

We study both detailed biophysical models and simplified models which are more amenable to analysis. Such an approach aids the isolation of the essential or minimal mechanisms underlying phenomena, the search for general principles, and the application of concepts and analogies from other fields.

We see a role for our group as intermediaries between the mathematical and biological disciplines. This includes disseminating the insights of mathematical work to biologists in accessible language and alerting mathematicians and other theoreticians to new and challenging problems arising from biological issues.

ANNUAL REPORT OF THE LABORATORY OF CELLULAR AND DEVELOPMENTAL BIOLOGY

The thirty-five members of LCDB focus on study of the critical elements of development and differentiation: how extracellular signals are translated, through transducers and second messengers, into differential gene expression in the nucleus, and how the architecture of nucleic acids and proteins is involved in nuclear and cytoplasmic function. This is an exciting area of modern biology and one in which members of the laboratory are widely recognized as key players.

Several noteworthy recognitions have come to laboratory personnel this year. Dr. Jurrien Dean spoke and chaired a session in a WHO conference on immunologic aspects of reproduction in Russia and at two conferences on reproductive immunology in Rome. Dr. Dean is a member of the NIH Biosafety Committee. Dr. Joseph Shiloach taught in a biotechnology course in Singapore and spoke at the Asia Pacific Biochemical Engineering Conference in Japan. Dr. John Louis Medabalimi spoke at the Workshop on HIV Drug Resistance in Amsterdam. Dr. Alan Kimmel spoke at an international meeting on Molecular Aspects of *Dictyostelium* Development and an AAAS symposium on The Revolution in Developmental Biology and serves as a member of the NIH Committee for Molecular Medicine. Dr. Andrew Greenberg received the Henry Christian Award for Excellence in Research of the AFRC. Dr. Robert Simpson assumed an Executive Editor position for *Nucleic Acids Research*, was a member of the review panel for the Gene Expression Programme of the European Molecular Biology Laboratory and taught in the Genetics Short Course at The Jackson Laboratory. He completed a three-year term as a member of the Molecular Cytology Study Section. Many members of the laboratory presented their work at other major meetings and lectured at NIH, universities and institutes.

My review of the laboratory's activities during the past year is not organized by Section or working group, but thematically. This approach provides the reader and us with an overview of the laboratory that emphasizes the continuity in research interests, a continuity which exists in spite of the broad span of areas of modern biology investigated in the Laboratory of Cellular and Developmental Biology.

MOLECULAR MECHANISMS OF GENE REGULATION

Nucleosome positioning has been suggested to be a possible mechanism in determining the function of *cis*-acting elements in eukaryotic cells. Previously, we showed that movement of a *cis*-acting element necessary for yeast replication origin function into the center of a nucleosome markedly diminished its accessibility to *trans*-acting factors. Similarly, we showed that a yeast repressor organized positioned nucleosomes abutting its operator, placing the TATA box in the center of the nucleosome, and suggested that this might be the mechanism of repression of the α -cell specific genes which are repressed by the $\alpha 2$ repressor protein. We have extended our investigations which indicated that a reporter gene linked to *MFA2*, an α -cell specific promoter, was active in certain amino terminal region deletions of histone H4. Now comprising over fifteen strains, the series of deletion and point mutants show absolute concordance between the absence of a precisely positioned, stable nucleosome abutting the $\alpha 2$ operator and the absence of full repression of the reporter gene. We conclude that the mechanism of repression of α -cell specific genes by the $\alpha 2$ repressor is establishment of a repressive chromatin structure wherein the TATA element is placed in an inaccessible location, in the center of a positioned nucleosome.

We have also examined the effects of chromatin structure on transcription of tRNA and 5S RNA genes by POL III. We previously reported that deletions in the 5' flanking region of the 5S RNA gene reduce *in vivo* synthesis of 5S RNA. Similarly, in constructs without deletions, moving the same region into a positioned nucleosome also reduces transcription; the data suggest that occlusion of the TFIIB binding site by interactions with histones in a nucleosome negatively affects transcription. In contrast, moving the TFIIB binding site into a predicted positioned nucleosome does not reduce transcription; whether a ternary TFIIB-DNA-histone complex is formed or whether the predicted nucleosome is absent remains to be established.

Another POL III gene, that for tRNA, behaves differently in the context of chromatin structure. Fusion of a tRNA gene to nucleosome positioning signals such that the tRNA start site and essential

A box element would be in a nucleosome led to no effect on transcription of the tRNA gene *in vivo* and absence of the predicted nucleosome. We have now found similar results for constructs which could place the 3' B box of a tRNA gene in a nucleosome; transcription is not affected. The suggestion from these results is that there is a competition in the cell between transcription factors and histones -- for POL III factors and tRNA genes, transcription wins but for POL III and 5S RNA or POL II, positioned nucleosomes can abolish transcription. It is possible that abundance of transcription factors may play a role in these differences.

The supposed mechanism for repression of α -cell specific genes by the $\alpha 2$ repressor, a repressive chromatin structure, raises the question of whether other repressors might function in a similar fashion. Like the $\alpha 2$ repressor, the engrailed protein of *Drosophila* represses transcription of several genes. Both these proteins are homeodomain proteins and they have essentially identical interactions with DNA. To test the possibility that engrailed also positions nucleosomes over promoters, the *MFA2* gene of yeast, controlled by $\alpha 2$, was replaced with a *MFA2-lacZ* fusion containing an engrailed binding site in place of the $\alpha 2$ operator. Placing an engrailed gene, under galactose control, in this strain should allow a direct examination of the hypothesis.

Protein DNA interactions at the $\alpha 2$ operator (and other specific loci) *in vivo* are of high interest in terms of understanding all the factors that interact with DNA and for comparison with structures deduced from *in vitro* reassembly using purified components. As one example of the problems faced in such studies, we have been unable previously to obtain a DNase I footprint of the $\alpha 2$ repressor bound to its operator due to the very short half life (<5 min) of the repressor. We have now overcome this problem by use of a plasmid to overexpress the $\alpha 2$ protein in a background of a deficiency in the ubiquitin mediated protein degradation pathway. Mapping of the $\alpha 2$ operator of the genomic *STE6* gene shows DNase I protection over a broader range than that found in *in vitro* complexes of $\alpha 2$ and MCM1 with naked DNA. Cutting sites within the operator are very different for α and α cells.

We have tried two additional approaches to determination of protein DNA interactions in living cells. In the first, we tried to express DNase I under control of a galactose promoter in yeast. Expression of wild type DNase I did not lead to cutting of plasmid DNA. When present on multicopy plasmids, addition of the SV40 signal sequence to target the gene product to the nucleus is apparently lethal; the only transformants recovered had rearrangements which deleted or truncated the nuclease gene. The construct was not lethal when on a centromere containing, single copy plasmid, however, little cutting of plasmid DNA was detected. There seems to be a delicate balance between cutting activity that can be compensated for by endogenous ligase and cutting activity that kills the cell.

The second approach uses another enzyme to probe chromatin structure. Others have previously utilized prokaryotic *dam* methyl transferase to modify DNA in *S. cerevisiae*. We have carried out a systematic investigation of the accessibility of DNA to the methylase. We constructed a derivative of the well characterized TALS plasmid containing eight GATC sites -- one in a hypersensitive region, one in a linker, and six placed at various locations within a positioned nucleosome abutting the $\alpha 2$ operator. A novel quantitative slot blot assay was developed to measure the extent of methylation at each site. The linker region is accessible in either α or α cells. The level of *dam* accessibility declines markedly in α cells 28-48 bp from the edge of the nucleosome and sites near the pseudodyad of the core particle are completely refractory to even hemimethylation in α cells. These same sites are frequently dimethylated in α cells, where the $\alpha 2$ repressor is absent as is the positioned nucleosome. The GATC sequence in the ARS1 origin of replication is methylated in about half of the minichromosomes. Although this is a nuclease hypersensitive region, it is apparent that other proteins, besides histones, can prevent access of the methylase to DNA; presumably, in this case it is the recently identified origin recognition complex. These results are of importance in establishing a benchmark for use of this powerful approach to study of chromatin structure *in vivo*.

Other studies in the laboratory address directly the role of *cis*-acting DNA elements and *trans*-acting factors in regulation of gene expression. The most studied system in this context is the heat shock gene *HSP82*, a gene whose expression is also induced during meiosis. Experiments in the last year, using a single copy *HSP82-lacZ* fusion integrated into the genomic *HSP82* locus, have defined the DNA sequences required for meiotic induction of the gene. Position of the gene does not seem to

effect its expression as similar levels of basal and induced expression, are observed when the reporter construct is located near a telomere (*HSP82* locus) or near a centromere (*LEU2* locus). Experiments comparing diploids and haploids, as well as those using *ime1* (inducer of meiosis) null strains demonstrate that induction of *HSP82* expression is a programmed developmental response.

A 250 bp segment of the 5' flanking sequence including the TATA box and a heatshock element (HSE) allow sporulation induction of transcription of a reporter gene. Point mutations in either element decrease both basal and sporulation induced expression; the specificity and relative magnitude of induction on sporulation are unchanged in these mutants. A forty bp region between the TATA box and HSE appears to contain a repressor element. Mutations in this region lead to a two- to fourfold elevation in basal expression in both haploids and diploids; sporulation induced expression is manifest in diploids with such mutations. A CNA motif with high homology to a repressor element found in a number of meiotic genes as well as in several general metabolism genes is present in this region. Dissection of the *cis*-acting elements important for the sporulation expression of the *HSP82* gene will continue as a prelude to the search for the *trans*-acting factors that mediate this response.

A number of mammalian genes whose expression is developmentally regulated are under investigation in the laboratory. One of these is the human epsilon globin gene, the earliest expressed of the genes in the beta-like globin gene cluster. Using DNase I footprinting and gel mobility shift assays, we have previously located binding sites for general and erythroid specific factors in the promoter of the epsilon globin gene. Similar studies with the beta globin locus control region (LCR) enhancer have identified binding sites for previously known factors as well as an uncharacterized factor, which we call epsilon1, and which binds at the promoter as well.

We have used clustered point mutations in globin gene regulatory elements fused to a CAT reporter gene to study the functional importance of the protein binding sites in the ϵ globin promoter and in erythroid specific enhancers. Our previous studies identified a GATA-1 site in the ϵ globin gene promoter (position -165) that was required for response of the promoter to two erythroid specific enhancers, the human LCR HS II enhancer, and the chicken β/ϵ globin 3' enhancer. However, GATA-1 sites in the enhancers were not required. The enhancers depended instead on AP-1/NF-E2 sites to effect transcription from this promoter. These results provide the best evidence to date that GATA-1 and AP-1/NF-E2 may interact with each other, either directly or indirectly. Thus, as few as two transcription factors, interacting through two sites on the DNA, may be required to establish enhanced transcription *in vivo*.

Expression studies suggested that in the absence of an enhancer GATA-1 did not act as a transcription factor for the ϵ globin gene. Since expression *in vivo* was low in the absence of the enhancer, we used *in vitro* transcription of the templates constructed for expression studies to further explore this question. Abundant transcription was directed by the naked DNA templates in erythroid as well as non-erythroid cell extracts. This presumably reflects the lack of tissue specific repression of naked DNA templates. The presence of the enhancer did not further increase transcription. Under these conditions, abolition of the -165 GATA-1 site in the promoter did not diminish transcription. Therefore, GATA-1 binding at this site serves to mediate interaction of the promoter with the enhancer and is not involved in promoter dependent transcription.

We used inhibition by oligonucleotides containing transcription factor binding sites to further study the requirements for *in vitro* transcription. GATA-1 was not required for transcription under these conditions. Sequestration of factors binding to the CACCC, CCAAT and TATA motifs by oligonucleotides containing these sequences severely inhibited transcription from the canonical cap site of the ϵ globin gene, although transcripts arising from alternate initiation sites in vector sequences were observed. Although AP-1 and Sp-1 can bind at these sequences in the ϵ globin gene, both addition and subtraction studies indicated that these factors are not involved in transcription of this gene promoter on naked DNA.

We have designed an episomal vector system which has potential to reveal much about the environment of the gene in chromatin in different transcriptional states. The vector is based on an EBV origin of replication, and contains the EBV nuclear antigen (EBNA-1) as well as a 5kb segment

containing the chromosomal ϵ globin gene marked by a two base pair mutation in the 5' non-coding region. The mutation produces a new restriction enzyme site. Clones of K562 cells and HeLa cells have been obtained following electroporation, that stably maintain 2-150 copies of the episome per cell. Eight K562 clones, and six HeLa clones were selected for further study. RNA was transcribed only from the genomic copy of the ϵ globin gene; no transcription from the minichromosomal gene was detected in either K562 or HeLa clones. This vector is "neutral" for expression of the ϵ globin gene.

The minichromosome was assembled into nucleosomes in the region of the ϵ globin gene insertion. This suggests that the gene may require its own enhancer to be expressed even in an erythroid environment; the simple availability of the erythroid transcription factors was insufficient to allow transcription. New minichromosomes containing either a μ LCR fusion, or the LCR HS II enhancer fragment will be used to test the effect of these additional sequences on transcription of the minichromosomes and on chromatin structure in the region of the ϵ globin gene.

Genes encoding the proteins which form the zona pellucida that surrounds growing oocytes, functions as primary and secondary sperm receptors and protects the preimplantation embryo are tissue specific and developmentally regulated. Two of these three genes, *ZP2* and *ZP3*, have been cloned and studied in this laboratory from both human and murine sources. Putative regulatory *cis*-acting sequences have been shown to be conserved for both genes in both species and necessary for the developmentally regulated expression of the zona genes. A 12 bp DNA motif about 200 bp upstream of the start site for transcription is necessary and sufficient for transcription of reporter genes microinjected into growing mouse oocytes.

In gel mobility shift assays, we have identified a putative transcription factor (*ZAP-1*) that binds to this DNA motif. Mutation of the binding site in the promoter of mouse *Zp-2*, mouse *Zp-3* or human *ZP2* dramatically affects promoter activity in reporter gene constructs microinjected into growing mouse oocytes. We presume that binding of *ZAP-1* plays a role in the coordinate and oocyte specific expression of the zona genes. The *ZAP* DNA complex is present only in growing oocytes and its appearance parallels that of *ZP2* and *ZP3* transcripts. The complex is detected in mouse, rat and human ovaries; it is not present in *Xenopus* oocytes (which do not have a zona pellucida). A concatenated oligonucleotide containing the binding site of *ZAP-1* has been used to screen a λ gt11 expression library. Nine positive clones have been identified and are currently being evaluated.

CELL BIOLOGY OF DEVELOPING SYSTEMS

While certain aspects of LCDB research also concern molecular levels of gene regulation, they are more focussed on the organismic aspects of development as a long term goal for understanding. In this area, studies of the development of *Dictyostelium* and studies of organ development, particularly gonadogenesis in the mouse, are examples of our research.

Mouse gestation is complete 20 days after fertilization. The primordial germ cells, first identified 7.5 days post-coitum (dpc), complete a migration from the allantois to the genital ridges by 12.5 dpc. The expression of the *Sry* gene (testis determining factor on the Y chromosome) in support cells beginning at 10.5 dpc is associated with the differentiation of the gonad into the testis by 12.5 dpc. In XX female mice, ovaries can be detected morphologically by 13.5 dpc.

A subtractive cloning strategy is being employed to identify genes that are either causal or a result of the differentiation of the primitive gonad into the ovary. Genital ridges from 10.5 to 12.5 dpc fetuses were isolated by dissection. The ridges were separated into male and female pools after their sex was determined by PCR using oligonucleotides specific to the *Sry* gene. Poly(A) RNA was isolated from each pool and 1 μ g of RNA used to construct separate plasmid based cDNA libraries. The female library contained 1×10^7 independent clones and the male library contained 4×10^6 . Clones from each library were amplified and single stranded phagemids from the male library were used to subtract out genes common to the male and female gonads. Investigations are underway to identify and characterize female specific clones. This strategy, while requiring a great deal of work, is a rational approach to study of genes important in organogenesis; study of the reproductive system has the virtue that mutations in genes necessary for organ formation will not be lethals, as they

would for most other organ systems.

Cyclic AMP is an important, pleiotropic effector of development for *Dictyostelium*, serving as a paracrine hormone. Development is dependent upon signal transduction mediated by cell surface, cAMP receptor/G protein linkages. Secreted cAMP acts extracellularly as a chemoattractant and primary signal to control cell motility, aggregation (multicellularity), cytodifferentiation, pattern formation and cell type specific gene expression. We have previously reported the isolation of four genes for distinct cAMP receptor subtypes, CARs 1, 2, 3 and 4. These genes have likely diverged from a common ancestor and comprise an entire cAMP receptor family. Each gene is expressed with distinct temporal and spatial patterns during the *Dictyostelium* developmental cycle.

The different cAMP receptor subtypes are expressed sequentially during development in the order CAR1, CAR3, CAR2, and CAR4. The relative affinities of the various subtypes for cAMP vary by ~100 fold with that for CAR1 > CAR3 > CAR2 > CAR4. During development, the levels of extracellular cAMP rise 100 fold, suggesting a distinct role for each subtype as cAMP levels change. Disruption of each gene by homologous recombination has now been carried out to address the contribution of the individual CARs in the developmental program. The major stages of *Dictyostelium* development are chemotaxis and cAMP signal relay, aggregate formation, formation of tipped structures as differentiated cells sort from one another, elongation to form a migrating slug of defined cell patterns and, finally, culmination and terminal differentiation. Each gene disruption yields a unique developmental phenotype which correlates closely with its temporal and spatial pattern of expression and affinity for cAMP. *CAR1* null cells fail to chemotax and aggregate; *CAR3* nulls are affected in their multicellular structures; *CAR2* nulls cannot form a tip and sort differentiated cell types; *CAR4* nulls have defects in pattern formation and late developmental events.

Surprisingly, all of the known responses of cells to cAMP cannot be explained simply by cAMP receptor subtypes 1-4. cAMP regulated gene expression is still observed in the receptor mutant cells. Yet, extensive DNA blot hybridization, cDNA and genomic library screening, PCR analysis, etc. indicate that CARs 1-4 comprise an entire gene family. Several genes which are related, albeit very distantly, to the known *CAR* genes have now been isolated. These new genes are likely to represent a distinct class of receptors that may also bind cAMP.

Characterization of the promoter regions for each of the cAMP receptor genes has been a major effort. Initial efforts have concentrated on the promoters of *CAR1* and *CAR3*. *CAR1* uses two promoters that are active at different developmental stages and that are differentially sensitive to extracellular cAMP stimulation. The early promoter is active during chemotaxis and its expression is induced by nM pulses of cAMP, whereas the late promoter is active at multicellular aggregation in response to higher, sustained concentrations of cAMP, a condition that represses the early promoter. In addition, the *CAR1* early promoter possesses a negative element that blocks expression in growing cells. Deletion analyses have localized the regulatory regions within several hundred nucleotides. For *CAR3* we have defined two separate elements within 80nt that are required for developmental regulation. Deletion of either completely blocks regulated expression of *CAR3*. Each promoter has been fused to the *E. coli* gene *lacZ* to examine receptor promoter activity in distinct cell types throughout the developmental cycle.

Many proteins involved in the regulation of eukaryotic transcription possess common structural features. Two of these domains, zinc fingers and leucine zippers, are present in a putative protein, ZnZ, whose gene has been identified in *Dictyostelium*. As an initial step in determination of the role of the ZnZ protein, we have disrupted its gene. In the mutant cells, the first half of development appears normal, but, as the slug begins to form, the morphology of the organism is progressively more aberrant and culmination is severely impaired. This suggests a critical role for ZnZ during late development, the time when the gene is maximally expressed.

LIPID METABOLISM, LIPASES AND GENETIC DEFECTS IN BOTH

A connection between two major segments of the laboratory, those studying developmental biology at the molecular level and those interested in lipid metabolism, has been made in the past few years;

the bridge was discovery of an adipocyte specific, hormonally responsive protein, perilipin, and cloning of its gene. Although the surface of the lipid storage droplet in adipocytes is the site of both deposition and retrieval of stored lipid, little if anything is known of the molecular details of reactions which occur at this critical juncture. Perilipin is associated with the lipid storage droplet. The cDNA for one form of perilipin (perilipin A; 57 kDa) was cloned from a rat cDNA library. More recently, with probes based on the cDNA of perilipin A, we have found a second cDNA, termed perilipin B, which encodes a protein of 46 kDa. The A and B forms of perilipin have a common amino terminus, but differ in their carboxyl terminal regions; the B form has only 3 of the 6 consensus A kinase phosphorylation sites found in the A form. Both forms of perilipin appear to arise from a common gene by alternate splicing of mRNA and both are found in mature rat adipocytes at the surface of the lipid droplet. Despite their resistance to solubilization in a variety of chaotropic agents and detergents, both primary and secondary structure analysis suggest minimal to modest hydrophobic composition. Interestingly, the perilipins appear not to contain regions of strong amphipathic helicity characteristic of other lipid binding proteins, such as the apolipoproteins and the plant lipid droplet proteins, oleosins. To further study the role of perilipin, a variety of constructs have been made which place perilipin in the sense or antisense orientation under control of promiscuous or hormone controlled promoters.

Previously we found that biotin deprivation did not reduce perilipin synthesis in cultured 3T3-L1 adipocytes, indicating that protein abundance is unrelated to lipid mass. However, in those studies removal of biotin from the culture medium merely retarded lipid synthesis; with time the cells deprived of biotin synthesized nearly as much lipid as did control cells. In the biotin deficient cells perilipin was found associated with many minuscule foci of lipid; we speculated that there was a relationship between perilipin and the aggregate lipid droplet surface area. Subsequently, we found that both biotin removal and avidin addition were necessary to block lipid synthesis in differentiating cells. Under such conditions, perilipin synthesis was at least equal to, and frequently greater than, that in control cells. These results indicate that lipid synthesis is not necessary for perilipin expression, and suggest that lipid packaging entities, perhaps the precursors to lipid storage droplets, are present prior to lipid synthesis.

Since perilipin is by far the most abundant A kinase substrate in adipocytes and since it is phosphorylated in concert with the lipolytic reaction, we speculated that the protein is an integral part of the lipolytic response mechanism, i.e., the lipid droplet surface is not a passive participant in lipolysis. Evidence for a change in the lipid droplet structure was obtained upon activation of 3T3-L1 adipocytes with the hydrolysis resistant cAMP analogs, 8-thiomethyl- and N⁶-benzoyl-cAMP. Together these compounds maintain A kinase in a chronically active state. Under phase microscopy, a change in the lipid droplet surface was evident within 2-3 hours after analog addition; droplet disruption was maintained for several days. Within 3 to 6 hours after analog withdrawal, the lipid droplets returned to the control state. Immunofluorescence with antibodies to perilipin using confocal microscopy, revealed a massive and dramatic alteration in the lipid droplet following analog activation. Whereas perilipin appeared as a uniform and compact shell around lipid droplets in control cells, a diffuse distribution of the protein was present in cAMP analog treated cells. Modification of the lipid droplet surface, perhaps to render the lipid accessible to the lipase, may be an important element of the lipolytic response. A further effect of cAMP stimulation, especially in 3T3-L1 adipocytes cultured in media supplemented with serum, was a rapid cessation of expression of both perilipin and hormone sensitive lipase. These data suggest that lipolytic stimulation may be self limiting.

Two other proteins of importance in lipid metabolism have been studied in this laboratory for many years, lipoprotein lipase (LPL) and hepatic lipase (HL). Studies have been carried out in tissue culture and *in vivo* in both normal mice and mice with *clt/clt* combined lipase deficiency. Lipoprotein lipase is necessary for hydrolysis of plasma triacylglycerol. The lipase is synthesized and secreted by parenchymal cells and transported to the luminal surface of capillary endothelium where it acts. Active LPL is a dimer of identical subunits with two N-linked oligosaccharide chains per subunit. In cultured mouse brown adipocytes and 3T3-L1 adipocytes, production of active LPL involves synthesis and glycosylation of LPL subunits in endoplasmic reticulum (ER), trimming of glucose and mannose residues from oligosaccharide chains in ER and Golgi, addition of other sugars to the chains in Golgi, dimerization of subunits, and secretion of lipase with complex type chains.

We have utilized chemical inhibitors and the genetic lesion in *clt/clt* mice to probe the relationship of glycosylation to activity, dimerization and secretion of LPL. The genetic defect in *clt/clt* mice is a recessive mutation on chromosome 17 which causes severe functional deficiencies of LPL and HL, extreme hyperlipemia, and death within 3 days in newborn mice. The structural genes for LPL and HL, located on chromosomes 8 and 11 respectively, are normal in *clt/clt* mice. Brown adipocytes cultured from *clt/clt* mice synthesized normal sized LPL which was glycosylated and partially processed, but the lipase was inactive, retained in ER, and present as aggregates of subunits. The LPL subunits contained endo H-sensitive oligosaccharide chains.

Our earlier studies showed that blocking glycosylation of LPL with tunicamycin resulted in synthesis of LPL which was inactive and retained in ER. Recently we found that the unglycosylated LPL subunits in tunicamycin treated adipocytes were present as aggregates. Normal processing of oligosaccharide chains is initiated by the removal of the outer glucose residue by the action of glucosidase I in ER. We found that blocking glucosidase I with castanospermine (CSTP) resulted in production of unprocessed (endo H-sensitive) LPL which was inactive, undimerized, and retained in ER. The intracellular site of dimerization of LPL is not known. Studies elsewhere indicate that dimerization of most proteins occurs prior to their exit from ER. The above findings suggest, however, that retention in ER may prevent dimerization of LPL subunits. This possibility was explored by treating cells with brefeldin A (BFA), a substance which can redistribute cis and medial Golgi proteins to ER. Treatment with BFA for 2 h resulted in formation of active dimeric LPL in CSTP blocked and *clt/clt* adipocytes, and formation of inactive dimeric LPL in tunicamycin blocked adipocytes. The latter indicates that core glycosylation is not required for dimerization of LPL subunits. Oligosaccharide chains of LPL subunits in CSTP blocked and *clt/clt* cells were processed to partially endo H-resistant forms in cells treated with BFA. The findings indicate that dimerization of LPL subunits requires some component(s) of cis/medial Golgi or the intermediate compartment between ER and Golgi. Thus, dimerization of LPL may normally occur in Golgi or some post ER organelle.

Lack of dimerization of LPL in tunicamycin treated and castanospermine treated cells probably resulted from inability of the ER to export unglycosylated and fully glycosylated proteins. The LPL subunits synthesized in *clt/clt* adipocytes, in contrast, were glycosylated and processed. Hence, retention of LPL subunits in ER in such cells probably resulted from a direct effect of the *clt* mutation on transport of LPL to Golgi. The above findings demonstrate that glycosylation and dimerization of LPL subunits are both required for activity of LPL. Our earlier findings with 1-deoxymannojirimycin, an inhibitor of Golgi mannosidase I, showed that processing of LPL subunits to partial endo H-resistance is not necessary for activity, dimerization or secretion of LPL.

The effect of chemical inhibitors and combined lipase deficiency on synthesis, activity and secretion of HL and LPL were studied in cultured hepatocytes from 1 day old mice. Normal hepatocytes synthesized and secreted active HL and LPL, and tunicamycin and CSTP decreased secretion of both lipase activities. Secreted HL was endo H-resistant, whereas intracellular HL was mostly endo H-sensitive. Intracellular LPL was also mostly endo H-sensitive. HL and LPL activities were greatly decreased, and both lipases were mostly endo H-sensitive in cultured *clt/clt* hepatocytes. The *clt* mutation blocked secretion of LPL, but not HL, in hepatocytes; the mutation affects the lipases in different ways.

The effects of cytokines on lipases and other proteins has been suggested to be important in the cachexia of wasting illnesses. Previous studies in this laboratory have shown that both IL-6 and TNF inhibit adipogenesis and lipoprotein lipase activity in 3T3-L1 cells, and that TNF also induces the production of IL-6. We have examined early signalling events by measuring acute protein phosphorylations stimulated by these two cytokines in 3T3-L1 adipocytes to determine if they shared similar signal transduction pathways. Autoradiography of 2 dimensional gels revealed that in ³²P-loaded cells both cytokines stimulated the phosphorylation of a 44 kDa protein (p44), but two p84 proteins phosphorylated in response to TNF appeared to be different from a p84 protein phosphorylated in the presence of IL-6. TNF, but not IL-6, stimulated the phosphorylation of p53. Western blot analysis of one dimensional gels with antiphosphotyrosine antibodies further emphasized the differences between the cytokines. Whereas IL-6 increased the phosphotyrosine content of p44, p84, p94, and p130, only p44 was affected by TNF. Kinetic studies revealed different time courses for

the phosphorylation of the various proteins, with peak times varying between 1 and 15 min. These results suggest that TNF and IL-6 mediate their actions by different signalling pathways and that changes in protein tyrosine phosphorylations may be important in the actions of IL-6.

The location of hepatic lipase in a number of tissues in rat has been defined. Well characterized antisera against rat hepatic lipase from three different laboratories gave similar results. Hepatic lipase in liver was localized primarily in the space of Disse, associated with extracellular matrix at basal surfaces of sinusoidal epithelium and hepatocytes and at surfaces of non-parenchymal cells and lipoprotein sized particles. Hepatic lipase was also present in the walls of terminal branches of hepatic artery and vein. Little hepatic lipase was found in the lumen of sinusoids and other vessels. Hepatic lipase in heart was most prevalent in the extracellular matrix around capillaries and some was at the surface of cardiac myocytes. A striking finding was the presence of hepatic lipase in aorta, beneath the endothelium and in the adventitia. Immunoreactive hepatic lipase was absent in tissues from rats preinjected with heparin. Hepatic lipase in the space of Disse could act on chylomicron remnants prior to uptake by hepatocytes while lipase in subendothelial space could act on lipoproteins transported across endothelium.

In addition to protein translocation, lipid trafficking is an important feature of the dynamics of cells. We studied cholesterol accumulation in cells using filipin as a probe for unesterified cholesterol. Cytochemical studies on cultured normal human fibroblasts and those derived from patients with Nieman-Pick Type C disease showed that the Golgi plays a role in the intracellular trafficking of LDL derived cholesterol. Freeze fracture cytochemistry showed that normal cells incubated with LDL accumulated cholesterol in trans Golgi vacuoles and cis Golgi, suggesting a Golgi mediated transport route for cholesterol to the plasma membrane and to the endoplasmic reticulum. In contrast NP-C cells accumulate LDL derived cholesterol in trans Golgi cisternae suggesting that sluggish mobilization of cholesterol from this to other Golgi compartments may be, in part, responsible for the cholesterol metabolic lesion present in the mutant cells. We have recently shown with fluorescence microscopy that normal fibroblasts, cultured with LDL in the presence of progesterone, accumulate cholesterol in perinuclear lysosomes. Progesterone treated cells contain higher levels of unesterified cholesterol and lower levels of cholesterol ester than normal cells incubated with LDL alone. We can reverse the lysosomal cholesterol lipidosis produced in normal cells by removal of LDL from the medium and washout of progesterone from the cells. The cells then show a burst of cholesterol ester formation with concomitant decrease in unesterified cholesterol in lysosomes. This progesterone related, reversible inhibition of lysosomal cholesterol trafficking is a useful experimental means of studying intracellular cholesterol transport in normal cells. We have used this normal cell model to show that monensin, a compound that inhibits Golgi membrane traffic to the plasma membrane, secondarily retards cholesterol release from loaded lysosomes.

Enzymology and protein structure

Several proteins of high interest in their own right or as models for fundamental processes in biochemistry are studied in the laboratory. Dihydrofolate reductase (DHFR) is the target enzyme for antifolate drugs which are widely used in treatment of neoplastic and autoimmune diseases. Chicken DHFR has long been known to be activated by the chaotropic compound urea. Slight activation has been observed in the presence of guanidinium salts; this is likely an ionic strength effect since similar activation is observed with sodium or potassium salts. In contrast, greater activation by the chaotropic guanidinium salts than by potassium salts is observed for recombinant human DHFR.

An important series of steps in infection by HIV-1 are the proteolytic cleavage of the polyprotein translated from the polycistronic mRNA by HIV-1 protease (PR). A new series of investigations in LCDB addresses aspects of the activation, dimerization, mechanism and inhibition of this important enzyme. Like other aspartic acid proteases, retroviral PRs possess an active site containing two sequences of AspThrGly. Cellular aspartic acid proteases are monomers, but for HIV-1 PR a homodimer formation is required to form the active site. This feature clearly distinguishes the HIV PR from the cellular aspartic acid PRs, and can be used to design specific inhibitors of dimerization. The initial step in polyprotein processing is presumed to be the folding and dimerization of the Gag-Pol polyprotein to form the first active PR. Such polyprotein dimers can then be responsible for the release of the "active form" of the PR necessary for the processing of the Gag and the Gag-Pol

polyproteins to produce the necessary functional proteins for viral infectivity. In order to attempt studies of the inhibition of the PR at the polyprotein level, we have developed an expression system for the production of inactive polyprotein mimics of the PR primarily to study the detailed mechanism of the activation process. The structure-to-function relationship of the self-activation of the PR was analyzed by specific mutational analysis at the cleavage sites of the PR.

Wild-type and mutant forms of the HIV-1 PR containing flanking Pol region sequences were expressed as fusion proteins with the maltose-binding protein of the *E. coli* *malE* gene. The full-length fusion protein (FLFP = MBP- Δ Pol-PR- Δ Pol) was purified using amylose affinity and size exclusion columns for mechanistic studies. PR is highly unstable and degrades rapidly in solution. Based on an extensive analysis of different buffer systems, we have defined the components that are critical to maintain the stability of the PR in solution, allowing reliable studies on the kinetics and dimerization of PR.

Analysis of self-processing of PR begins with a basic construct consisting of the maltose binding protein (MBP) fused to the HIV-1 PR with flanking Pol region sequences containing the native cleavage sites. The chimaeric protease can assemble to form a fully active dimer. Fusion proteins containing mutations at positions immediately adjacent to the N- and C-terminal amino acids of the wild-type PR were constructed, expressed and purified. The N-terminal mutations, Phe \rightarrow Ile or Phe-Ala-Pro, and the C-terminal mutation Pro \rightarrow Ile resulted in loss of cleavage at the mutated sites and reduced cleavage at the non-mutated sites. In these mutants, after the primary cleavage at one of the non-mutated sites, there must be a conformational change which prevents the cleavage at the other non-mutated site. The parallels to the recently reported conformational change in HIV-1 reverse transcriptase are highly interesting. Ala insertion at the C-terminus does not substantially interfere with the assembly of active enzyme and cleavage at both termini. In contrast, Ala insertion at both ends prevented cleavage at both ends. Comparisons of the processing activity between clones suggested that an insertion mutation at the C-terminal site is tolerated, provided the wild-type site is preserved at the N-terminus. These results indirectly suggest that N-terminal cleavage precedes cleavage at the C-terminus.

For kinetic analysis of the autoprocessing reaction, purified FLFP was denatured in 5 M urea and renatured by dilution. The renatured fusion protein undergoes time dependent autoprocessing in parallel with the appearance of HIV-1 PR activity. The time course of the reaction was monitored by SDS-PAGE and staining for disappearance of the FLFP, appearance of the MBP and by immunoblotting to quantitate the appearance of the 13.2 kDa cleavage intermediate and 11 kDa mature PR. The appearance of enzymatic activity clearly coincides with appearance of the MBP, disappearance of FLFP and the sum of the 13.2 kDa cleavage intermediate which corresponds to the PR- Δ Pol' fusion protein and 11 kDa PR. This reaction was determined to be first-order from initial rate measurements of the appearance of enzymatic activity ($k_{act} = 0.044 \pm 0.003 \text{ min}^{-1}$), and the disappearance of the full-length fusion protein ($k_{dis} = 0.040 \pm 0.003 \text{ min}^{-1}$). This indicates that the FLFP exists mostly as a dimer under these conditions and that activation of the PR from the fusion protein occurs through an intramolecular cleavage at the amino terminus. Both the HIV-1 substrate and pepstatin A inhibit the autoprocessing reaction indicating that the substrate binding site is intact in the FLFP. Pepstatin A inhibits autoprocessing with K_i (0.21 μM) that is at least fivefold lower than K_i for the mature PR. The non-covalent dissociative inhibitor Ac-Thr-Leu-Asn-Phe-COOH of the mature PR at a concentration of 1 mM had no significant effect on the release of the 13.2 kDa cleavage intermediate which is enzymatically competent.

Characterization of the HIV-1 PR has been facilitated by a collaborative study with the Biotechnology Unit of LCDB; the scheme enables isolation of pure PR that is several orders of magnitude more active than that from any currently available commercial source. This has enabled us to conduct detailed analysis of (i) the effect of salt on the kinetic parameters of retroviral and mammalian aspartic acid proteases in collaboration with LBC, NIDDK and (ii) kinetic and modeling studies of S_3-S_3' binding sites of HIV proteinases in collaboration with the LMVC/ABL-BRI/NCI-FCRDC.

The three zona proteins are secreted and form an extracellular matrix surrounding growing oocytes. We have identified antisense oligonucleotides which, when microinjected into growing oocytes, cause

the specific degradation of either ZP2 or ZP3 transcripts. New zona protein synthesis of the targeted mRNA is abolished but the incorporation of the other zona proteins into the extracellular matrix is unaffected. Thus, the biosynthesis and secretion of the zona proteins appear to be independent of each other. Efforts are now underway to determine if heterologous human zona transcripts can be synthesized, post-translationally modified and integrated into the preexisting mouse zona pellucida. By creating the appropriate mutations we should be able to identify domains important for zona protein interactions in the zona matrix.

Barnase, an extracellular ribonuclease of *B. amyloliquefaciens*, and barstar, its intracellular inhibitor, are both small proteins which form a one to one complex and undergo two state physical transitions. Barnase has become the system of choice for protein folding experiments; a recent issue of *J.Mol.Biol.* had 13 articles using barnase as the experimental system. The barnase/barstar complex seems likely to become the paradigm for study of protein protein interactions in the same fashion that barnase is for folding. Both genes have been cloned, sequenced, expressed and subjected to directed mutagenesis. The barnase structure has been refined against 2.0 Å x-ray data. The solution (NMR) structure of barnase has been solved in another laboratory and agrees well with the crystal structure. The crystal structure of the barnase-barstar complex has now been solved in Dr. Yves Manguen's laboratory, using a mutant barstar in which the two cysteines have been replaced by alanines. The structure, which is currently being refined, shows a barstar made up primarily of four helices and a small three stranded β sheet, with one of the helices covering the active site of barnase. Solution of the NMR structure of both barstar and the complex is well along in several laboratories.

Three methods have been devised for measuring parameters of the barnase-barstar reaction for wild type and mutant proteins. The first is a simple titration of barnase activity by barstar and yields absolute dissociation coefficients for pairs with such coefficients greater than 10^{-11} M. The second measures the (equilibrium) binding of an active barnase to a barstar in the presence of a competing inactive barnase and yields a ratio of the two dissociation coefficients. The third measures the absolute off-rates for certain barnase-barstar pairs. If the on rates are assumed to be equal (e.g. diffusion limited) the second two methods should, and do, give the same dissociation coefficient ratios. By combining these methods on several different pairs, it has been possible to estimate the dissociation coefficient of the wild type pair at about 1×10^{-13} . We have also been able to compare the contributions to the binding specificity of Arg 59 and His 102 of barnase and the disulfide bond of barstar.

A new barnase plasmid vector has been devised, in which control of the gene is tight enough to allow us to carry wild type or active mutant barnase genes without expression of barstar and to carry active mutants which are not inhibited by barstar. In the normal (off) state of this vector a gene for the lambda antitermination (N) protein and the barnase gene are read backwards from a *tac-lac* promoter to produce antisense RNA which counters any adventitious reading of barnase in the other direction. The barnase gene is further inhibited by a 5' transcription terminator. With the two genes flanked by lambda *attP* and *attB* sequences, a heat shock turns on the host's *int* function and inverts them, turning on the N protein gene which in turn allows transcription of the barnase gene. The first uses of this system have been to detect low levels of activity (by their lethal effect on reversal) of mutants which were too unstable to produce detectable activity otherwise and to show that certain mutations produced barnases that were lethal because they were active but not inhibited by barstar. We think the system can now be used for direct selection of secondary mutations in either barnase or barstar that compensate primary mutations which interfere with inhibition. We can thus explore the interface between the two proteins as well as asking direct questions about protein protein interactions by finding compensatory mutants that suppress mutations that disrupted barnase barstar complex formation.

We have continued to distribute the model substrates for higher order chromatin structure that contain tandemly repeated nucleosome positioning DNA sequences to other laboratories; these molecules are currently in use in over a dozen other sites. Dr. Timothy Richmond at the ETH in Zurich, Switzerland, has nearly completed the determination of a high resolution X-ray crystal structure of the core particle of the nucleosome, using a unique DNA fragment based on the 5S rRNA positioning signal and constructed in this laboratory in a collaborative effort.

BIOTECHNOLOGY

Certain aspects of research done in the laboratory have actual or potential applications in the clinic or field. Barnase and barstar have been used by others to create male sterile plants and to reverse the sterility by expression of the inhibitor. This advance should have a major impact on the crop seed business; it may well be the most significant application of genetic engineering to agriculture thus far.

Knowledge of the molecular biology of the zona pellucida protein genes has allowed laboratory members to devise a logical approach to immunocontraception. A ZP3 peptide (amino acids 328-343) contains a B-cell epitope that when coupled to KLH is capable of eliciting antizona antibodies that prevent fertilization. The peptide also contains a T-cell epitope which in some, but not all, inbred strains of mice elicits autoimmune oophoritis. The T-cell (amino acids 330-337) and B-cell (amino acids 336-342) epitopes partially overlap. When amino acids 328-335 of the T-cell epitope are replaced with alanine residues, the ZP3 peptide no longer elicits autoimmune oophoritis even in susceptible strains of mice. This modified peptide (coupled to KLH to provide T-cell helper function) elicited sufficient antizona antibody to coat the endogenous zonae pellucidae in 3 out of 18 vaccinated animals. Thus, while it appears that the disease causing epitope can be separated from the antibody provoking epitope, additional investigations will be required to enhance the antizona antibody titers in all members of a population.

The Biotechnology Unit of the laboratory is a research and development facility in addition to providing fermentation and processing services for all of the NIH community. During the past year, the unit performed 212 large scale preparations including growth of microorganisms in volumes up to 300 liters and eukaryotic cells in volumes up to 50 liters. Gram quantities of a recombinant, modified *P. aeruginosa* exotoxin A were purified from cultures expressing the mutant gene. More than 20 grams of S antigen was purified from bovine retina for use in clinical trials using oral tolerance as an approach to autoimmune retinitis. In an effort to improve the efficiency of eukaryotic expression systems, a continuous perfusion process was developed that allowed a fivefold increase in density of HeLa cell cultures; vaccinia virus infection and expression of recombinant genes were equally efficient at either cell density.

This overview of the work of the Laboratory of Cellular and Developmental Biology is a brief summary of the studies carried out in the past year by talented and productive scientists. We hope that the connectiveness of our science and its diversity leads the reader to a sense of the excitement we feel in our daily interactions as we move forward in understanding of the most interesting aspects of cellular and molecular biology in a very exciting time for modern biology.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15100-22 LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Protein Nucleic Acid Interactions: Chromatin Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.:	Robert T. Simpson	Laboratory Chief	LCDB:NIDDK
Others:	Julia P. Cooper	Staff Fellow	LCDB:NIDDK
	Michael P. Kladde	Staff Fellow	LCDB:NIDDK
	Randall H. Morse	Senior Staff Fellow	LCDB:NIDDK
	Michael R. Murphy	IRTA Fellow	LCDB:NIDDK
	Sharon Y. Roth	Senior Staff Fellow	LCDB:NIDDK
	Christopher Szent-Gyorgyi	Senior Staff Fellow	LCDB:NIDDK

COOPERATING UNITS (if any)

T. Richmond, ETH, Zurich, Switzerland (foreign)

LABORATORY

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

6.5

PROFESSIONAL:

6.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

The role of chromatin structure in modulating the functions of DNA in transcription, replication, recombination and repair is becoming increasingly apparent. We have shown that the yeast $\alpha 2$ repressor positions a nucleosome adjacent to its operator. This chromatin structure seems to be the mechanism of repression; variant forms of histone H4 that lead to abolition of the structure also lead to expression of a reporter gene driven by the promoter. Hierarchies exist in the contest between transcription factors and histones. While $\alpha 2$ can organize chromatin structure and repress transcription, TFIIIA and factors necessary for transcription of yeast tRNA genes establish active transcription complexes and prevent the formation of stable nucleosomes. The *Drosophila* engrailed protein has strong homology in its homeodomain DNA binding region with yeast $\alpha 2$ repressor. Experiments designed to ascertain whether engrailed, expressed in yeast, can repress genes normally under control of $\alpha 2$ are underway, in an effort to address the generality of the phenomenon. Mapping of protein DNA interactions in situ has been a major effort in the past year. Use of yeast strains that increase the half-life of the $\alpha 2$ repressor has allowed elucidation of the structural features of the complex of the repressor, MCM1 and DNA at the $\alpha 2$ operator. Experiments using galactose controlled expression of DNase I in yeast have shown that there is a narrow cusp between no effect and lethality in expression of the gene and detectable DNA cutting. A systematic study of the use of prokaryotic dam methylase for probing chromatin structure has defined the accessibility of nucleosomal, linker, and hypersensitive region DNA to the modifying protein. Studies of the expression of the sporulation induced HSP82 gene have defined elements that are necessary for normal expression, although likely not for meiosis responsiveness, and an apparent repressor sequence. Collaborative studies of higher order chromatin structure and the crystal structure of a nucleosome core particle containing unique sequence DNA continue.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 15102-32 LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of a ribonuclease and its inhibitor from Bacillus Amyloliquefaciens

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.	Robert W. Hartley	Research Physicist	LCDB:NIDDK
	Milan Jucovic	Visiting Fellow	
	Yelena Chernokalskaya	Visiting Associate	

COOPERATING UNITS (if any)

Dr Yves Maugen, Lab de Physique, Centre Pharmaceutique, Univ de Paris-Sud.
 Dr Jean Garnier, Protein Engineering Unit, Biotechnology INRA, Jouy-en-Josas, France. Dr Guy Dodson, Chem Dept, U of York. Dr Josef Sevcik, Inst of Molecular Biology, Slovak Academy of Science, Bratislava, Czechoslovakia. Dr Genady Moiseyev, Inst of Molecular Biology, Russian Academy of Sciences, Moscow, Russia. Dr Franklyn Prendergast, Mayo Foundation, Rochester, Minnesota.
 Laboratory of Cellular and Developmental Biology

SECTION

Developmental Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.5

OTHER

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Two proteins, barnase, the extracellular ribonuclease of Bacillus amyloliquefaciens, and barstar, its intracellular inhibitor, are used as a model system for the study of protein folding and protein-protein interactions. Barnase is one of an homologous group of ribonucleases occurring in both prokaryotes and eukaryotes.

Recombinant DNA techniques are being applied with three major aims: (1) to facilitate production of wild type and mutant proteins; (2) to examine the structural and control sequences of the genes; and (3) to make specific changes in the sequences to test theories of folding and probe the barnase-barstar interaction.

The lethal effect of the cloned wild type barnase gene can be repressed by expression of the barstar gene on the same plasmid. *E. coli* plasmid vectors have been devised for both proteins and both can now be obtained essentially pure in 100 mg quantities. DNA and amino acid sequences and x-ray structures of both are known as well as the NMR solution structure of barnase. A synthetic fluorescent substrate has been used to study hydrolysis kinetics and to look at the kinetics and stability of the barnase-barstar interaction for native and mutant proteins. Close to a hundred directed mutations in each protein have been produced. Some of these were aimed at specific questions but most are part of a survey of the protein surfaces designed to locate their areas of interaction and residues on both have been identified as being so involved. The two Cys residues of barstar can both be replaced by Ala without loss of activity (*in vitro* or *in vivo*, but with some reduction in the strength of the bond) or yield will greatly simplify future studies of barstar folding. Such replacement of either or both of the Cys residues reduces the stability of barstar only to that of the wild-type measured in the presence of mercaptoethanol or DTT. Several methods have been developed for measuring the relative and absolute strength of the bond between barnase and barstar for various combinations of wild-type and mutant proteins. For the wild-type proteins the dissociation coefficient is on the order of 10 to the -13.

Recent work, elsewhere, in which the barnase gene was attached to a eukaryotic promoter in order to kill the tissue in which that promoter is expressed (in the first instance to produce male sterility in plants) has aroused considerable interest in its possible use in developmental studies and is the key to a variety of anti-viral strategies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15200-32 LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on Folic Acid (Dihydrofolate Reductase) and Vitamin A (Beta-Carotene)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Bernard T. Kaufman
 Others: John G. Bieri

Section Chief LCDB:NIDDK
 Scientist Emeritus LCDB:NIDDK

COOPERATING UNITS (if any)

Dr. J.C. Smith, U.S. Department of Agriculture, Beltsville, MD; Dr. James Freisheim, Medical College of Ohio; Dr. Michele McTigue, University of California at San Diego

LAB/BRANCH

Laboratory of Cellular & Developmental Biology

SECTION

Nutritional Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1

PROFESSIONAL:

1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrevoked type. Do not exceed the space provided.)

This project continues to focus on dihydrofolate reductase, a critical enzyme in the metabolism of the B-vitamin, folic acid. The maintenance of folic acid at the tetrahydro-level by dihydrofolate reductase is critical to cellular survival. Antifolate drugs which target dihydrofolate reductase continue to be widely used in the treatment of cancer, rheumatoid arthritis, and an increasing number of autoimmune diseases.

All vertebrate dihydrofolate reductases examined to date have the unique ability to have their catalytic activity significantly stimulated when treated with certain agents known to perturb the tertiary structure of proteins. Chicken liver (ckDHFR) dihydrofolate reductase shows a fivefold to sixfold increase in activity when assayed in the presence of about 5 M urea. Guanidinium (Gdn) salts are among the most interesting protein perturbants because of the strong denaturing activity usually associated with the Gdn ion. However, ckDHFR shows only minimal activation with relatively low concentrations of GdnHCl, i.e., approximately twofold at 0.2 M. Higher concentrations of GdnHCl results in rapid denaturation and loss in activity. Since this increase in activity is in the same range as observed in the presence of corresponding concentrations of Na or KCl, it is concluded that this stimulation is due to ionic or salt effects, rather than the well known chaotropic properties of Gdn compounds. However, the Gdn moiety is affecting the protein since equivalent or higher concentrations of salts do not cause similar denaturation despite corresponding activations. On the other hand, the recombinant human DHFR (rhDHFR) does appear to exhibit an activation in response to Gdn HCl. At 0.6 M GdnHCl, the rhDHFR shows about a 40% higher activation than the maximum activation observed with KCl. Similarly, the sheep liver DHFR exhibits a significantly higher activation with 0.65 M GdnHCl than activation induced by ionic strength. Additional studies with the thiocyanate, acetate, and sulfate Gdn salts revealed similar activations with the thiocyanate and acetate salts. However, GdnSO(4) was found to be a potent inhibitor of all of the DHFRs. Similar results were obtained with NaSO(4). The significance of these results are being interpreted in terms of the activating properties of urea and the known preferential interactions of proteins with the guanidinium ion and salts as well as recent results from x-ray studies on an activated ckDHFR. Studies continue to focus on beta-carotene, its putative antioxidant properties and its relationship to vitamin A. A variety of carotenoids, including beta-carotene, showed no *in vivo* antioxidant properties when fed to animals deficient in vitamin E and selenium. A copper deficiency had no effect on carotene metabolism in view of the fact that the carotene-converting enzyme, carotene epoxidase, requires copper.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15401-20 LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthesis and Transport of Lipoprotein and Hepatic Lipases in Cells and Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Robert O. Scow	Chief, Endocrinology Sect.	LCDB:NIDDK
Others:	Jin-Woo Park	Visiting Fellow	LCDB:NIDDK
	Charles J. Schultz	IRTA Predoctoral Fellow	LCDB:NIDDK
	E. Joan Blanchette-Mackie	Research Biologist	LCDB:NIDDK
	Albert E. Spaeth	Chemist	LCDB:NIDDK
	Do-Gon Ryu	Special Volunteer	LCDB:NIDDK

COOPERATING UNITS (if any)

Dr. Hiroshi Masuno, 2nd Department of Medical Biochemistry, School of Medicine, Shigenobu, Ehime, Japan; Dr. Tsuneo Takahashi, Department of Oral Anatomy, Kanagawa Dental College, Yokosuka, Kanagawa, Japan

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

4.0

PROFESSIONAL:

2.0

OTHER

2.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Mice born with combined lipase deficiency (cld/cld) have very low lipoprotein (LPL) and hepatic (HL) lipase activities, develop severe hypertriacylglycerolemia, and die within 3 days. The recessive mutation (cld) causing the deficiency is located on chromosome 17, whereas LPL and HL genes are on chromosomes 8 and 11, respectively. Structural genes for the lipases are normal in cld/cld mice. Both lipases are synthesized in parenchymal cells and transferred to endothelial cells where they normally act. The active form of LPL is thought to be a dimer of identical endo H-resistant glycopeptides. Brown adipocytes cultured from cld/cld mice synthesized normal-sized LPL subunits which were glycosylated and partially processed, but the lipase was inactive, retained in ER, and present as aggregates of subunits. The LPL subunits contained endo H-sensitive oligosaccharide chains. LPL was also retained in ER of cultured cld/cld hepatocytes. Blocking glycosylation of LPL with tunicamycin in normal adipocytes resulted in synthesis of LPL which was inactive, retained in ER, and present as aggregates. Normal processing of oligosaccharide chains is initiated by removal of the outer glucose residue by action of glucosidase I in ER. Blocking glucosidase I with castanospermine (CSTP) in normal adipocytes resulted in production of unprocessed (endo H-sensitive) LPL which was inactive, undimerized, and retained in endoplasmic reticulum (ER). These findings suggested that retention in ER prevented dimerization of LPL subunits. Treatment of cells with brefeldin A, which can redistribute cis and medial Golgi proteins to ER, resulted in formation of active-dimeric LPL in cld/cld and CSTP-treated adipocytes, and formation of inactive-dimeric LPL in tunicamycin-treated cells. The findings indicate that dimerization of LPL subunits requires some component(s) of cis/medial Golgi or the intermediate compartment between ER and Golgi, and that glycosylation and dimerization of LPL subunits are both required for activity of LPL. They also indicate that retention of LPL in ER of cld/cld cells probably results from a direct effect of the cld mutation on transport of LPL from ER to Golgi.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15404-08 LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ultrastructural Immunocytochemistry of Lipid Metabolism in Cells and Tissue

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: E. Joan Blanchette-Mackie
Others: Robert O. Scow
Nancy K. Dwyer
Robert A. Coxey
Therese Barber

Research Biologist LCDB:NIDDK
Chief, Endocrinology Sec LCDB:NIDDK
Biologist LCDB:NIDDK
IRTA, Post Doctoral Fellow LCDB:NIDDK
IRTA, Pre-Doctoral Fellow LCDB:NIDDK

COOPERATING UNITS (if any)

Dr. Peter Pentchev, Dev Metab. Neurol Branch, NINCDS, NIH; Dr. Howard Kruth Lab Exptl Ather, NHLBI, NIH; Dr. Constantine Londos, Lab Cell Develop Biol, NIDDK, NIH; Dr. Michael C. Scholtz, Dept. of Medicine, U. California, CA.

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda Maryland 20892

TOTAL STAFF YEARS:

4.0

PROFESSIONAL:

2.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreserved type. Do not exceed the space provided.)

We located hepatic lipase in space of Disse and subendothelial space of heart and aorta. Hepatic lipase in the space of Disse was associated with the extracellular matrix at basal surfaces of sinusoidal epithelium and hepatocytes, and at surfaces of non-parenchymal cells and lipoprotein sized particles. Hepatic lipase was present in heart in extracellular matrix around capillaries and at surfaces of myocytes. A striking finding was the presence of hepatic lipase in aorta, beneath the endothelium and in the adventitia. Thus hepatic lipase was located in the space of Disse, where it could act on remnants prior to uptake by hepatocytes, and in subendothelial space, where it could act on lipoproteins transported across endothelium.

Perilipin, a hormonally regulated phosphoprotein, was located intracellularly in cultured 3T3-L1 adipocytes, adipocytes isolated from rat epididymal fat pads and adipocytes within lactating rat mammary gland. In developing adipocytes and mature adipocytes, perilipin is localized on the monolayered surface of lipid droplets. Perilipin was not found in mammary alveolar cells which synthesize and secrete triacylglycerol lipid droplets as milk lipid. Immunofluorescent studies on cultured 3T3-L1 adipocytes incubated with cAMP analogues show, that cells decrease perilipin at the lipid droplet surface and disperse perilipin into the cytoplasm in response to cAMP kinase activation.

Golgi compartments are involved in the intracellular trafficking of cholesterol derived from LDL. In normal fibroblasts both trans Golgi vacuoles and cis Golgi cisternae accumulate cholesterol in response to LDL uptake suggesting a route of transport for cholesterol from trans Golgi vacuoles to plasma membrane and cis Golgi to endoplasmic reticulum. In fibroblasts derived from patients with Niemann-Pick Type C disease cholesterol accumulates in trans Golgi cisternae suggesting impaired cholesterol transport through the Golgi. The ability of cells to process cholesterol rich lipoproteins could depend on modulation of cholesterol enriched membrane traffic through the Golgi to intracellular homeostatic sites. We found recently that normal fibroblasts, incubated with progesterone and LDL abnormally accumulate cholesterol in lysosomes. Upon progesterone removal, unesterified cholesterol leaves lysosomes and cholesterol ester is synthesized in these normal cells. However, monensin, a compound which inhibits Golgi membrane trafficking to the plasma membrane, retards cholesterol efflux from cholesterol laden lysosomes and increases cholesterol in Golgi. This rapidly reversible cholesterol lipidosis in normal cells provides an experimental opportunity to study mechanisms that regulate intracellular cholesterol transport.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15500-31-LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Large-Scale Production and Purification of Compounds with Biological Activity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Joseph Shiloach
Others: Jeanne B. Kaufman
Yong-Jick Kim
Amos M. Tsai

Research Chemist
Biologist
Visiting Fellow
Guest Researcher

LCDB:NIDDK
LCDB:NIDDK
LCDB:NIDDK
LCDB:NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Biotechnology Unit, Office of the Chief

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3

PROFESSIONAL:

2

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

The Biotechnology Unit is responsible for:

- Large-scale production of procaryotes.(bacteria), and eucaryotes (mammalian cells, insect cells).
- Large-scale recovery and purification of biologically active compounds (proteins, polysaccharides, etc.) from various sources.
- Process development work associated with (1) bacterial growth, (2) eucaryotic cell growth, and (3) extraction and purification of biologically active compounds, especially proteins. The process development work is conducted to develop a procedure suitable for large-scale preparation and production of material suitable for clinical trials.
- Research and development work not necessarily linked to a current process development project, but work that has long-term implications for fermentation processes and protein purification.

During the last year the Unit performed 212 different large-scale preparations, including microorganisms (especially E. coli-carrying recombinant DNA) grown in volumes ranging from 5 to 300 liters, eucaryotic cells grown in volume up to 50 liters and processing of various biological materials.

Special effort was devoted to the development of a suitable process for the extraction and purification of large amounts of two proteins needed for clinical trials. The first is S antigen from the human retina and the second is a modified toxic version of Pseudomonas aeruginosa exotoxin A.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15503-11 LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Regulation of Developmental Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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	Jasmine Gruia-Gray	Visiting Fellow	LCDB:NIDDK
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COOPERATING UNITS (if any)

Drs. Andrew S. Greenberg and Constantine Londos, LCDB
 Dr. Peter Devreotes, Johns Hopkins School of Medicine

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PROFESSIONAL:

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CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard, unenriched type. Do not exceed the space provided.)

Dictyostelium development is regulated by response to secreted cAMP. We have isolated genes for 4 cAMP receptor subtypes and determined their role during the Dictyostelium developmental cycle. Each has a distinct affinity for cAMP which correlates well with the relative concentrations of extracellular cAMP present at the time of their expression. Dictyostelium specifically disrupted for each gene exhibit developmental abnormalities consistent with their cAMP affinities and temporal and spatial patterns of expression. Thus, the multiple responses of cells to cAMP can be attributed to distinct receptor subtypes encoded by unique genes. Nonetheless, current data suggest that cAMP receptors, other than CARs 1-4, play a role during Dictyostelium development. Mutated Dictyostelium are still able to regulate gene expression by cAMP-dependent event. We have now isolated several genes which share weak sequence homology with the known cAMP receptor genes across their entire coding regions. These genes potentially encode a new set of cAMP receptors which may mediate cAMP regulated gene expression. Alternatively, they may interact with a completely different ligand; several other G-protein-linked responses have been described in Dictyostelium. The promoters which regulate the expression of the cAMP receptor genes have been identified. Deletion studies indicate that they may be under both positive and negative control and in some cases we have localized specific DNA elements that are absolutely required for developmentally regulated expression. Interestingly, the cAMP receptors themselves are regulated by response to extracellular cAMP. In particular, subtype 1 uses a dual promoter system. Each is active at a distinct developmental stage and responsive to a different signalling mechanism. The promoters have been fused with reporter genes that permit the detection of cell-type specific expression patterns. Consistent with our previous studies of mRNA localization, the promoters exhibit specific patterns of localized expression. The mammalian adipocyte represents another excellent system for the study of differentiation. We have primarily focussed on the structure and function of perilipin, an adipocyte-specific, phosphoprotein that is localized at the periphery of lipid droplets. We have isolated cDNA and genomic sequences for perilipin from rat and mouse. Molecular analyses predicted the existence of two perilipin protein forms that result from alternative RNA splicing; these protein forms have been confirmed. Expression in sense and antisense orientations is being used to examine the function of perilipin in lipid metabolism.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK :5505-14 LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Adipocyte Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

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A.R. Kimmel, J. Blanchette-Mackie, J. Gruia-Gray, LCDB:NIDDK; R.P. Nordan, DCT:NCI; P. Coon, A.P. Goldberg, U. Maryland, Baltimore; R.H. Pointer, Howard University; N. Edens, Rockefeller University, G. Michaels, DCRT.

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4.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Two areas of research on hormonal control of adipocyte metabolism are summarized: (A) Previously, we reported the discovery of perilipin, an adipocyte-specific protein that is associated with the lipid storage droplet and multiply phosphorylated upon elevation of cAMP. We now have obtained cDNAs that contain the entire predicted coding sequences for major (57 kDa) and minor (46 kDa) forms of perilipin, A and B, respectively. The two forms appear to arise from alternative splicing, and both proteins are found in rat adipocytes in tight association with the lipid storage droplet. Total blockade of triacylglycerol synthesis does not interfere with perilipin expression, suggesting that nascent lipid droplets, the lipid packaging moieties, arise independent of lipid synthesis. Confocal microscopy combined with immunofluorescence with anti-perilipin antibodies reveals that lipolytic stimulation of cultured 3T3-L1 adipocytes leads to a massive structural change in the lipid storage droplet surface. Such data indicate that the lipid droplet is an active participant in lipolysis, i.e., activation of the rate-limiting enzyme, hormone-sensitive lipase, is but one of several concerted reactions required to mobilize the stored lipid. (B) Previously, we found that Interleukin-6 (IL-6) acts directly on adipoblasts to inhibit their differentiation into adipocytes and on adipocytes to inhibit lipoprotein lipase activity, data that suggest a role for this cytokine in cachexia. Similarly, tumor necrosis factor (TNF/cachectin), another factor implicated in cachexia, acts directly on adipocytes to stimulate IL-6 production. A comparison of the effects of IL-6 and TNF in radiophosphate-loaded 3T3-L1 adipocytes by autoradiography of 2D gels reveals that the cytokines stimulate the phosphorylation of different proteins. Similarly, IL-6 stimulates the tyrosine phosphorylation of 4 different proteins, only one of which is modified by TNF. These data indicate that the two cytokines act by different signalling mechanism and suggest that tyrosine kinase activation may mediate some IL-6 effects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15506-08 LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Gene Expression in Mammalian Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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COOPERATING UNITS (if any)

Zhi-Bin Tong and Larry Nelson, DEB, NICHD; Kenneth Tung, University of Virginia, Charlottesville, VA; Simon Kiperzstok, George Washington University Medical School, Washington, DC.

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PROFESSIONAL:

5.5

OTHER

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- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

The mouse ovary serves as a paradigm for investigating the developmental biology of mammalian gonadogenesis, oogenesis and fertilization.

Gonadogenesis: Mouse gestation occurs over 20 days. Germ cells, first detected in the developing embryo 7.5 days post coitus (dpc), migrate from the allantois to the genital ridge by 12.5 dpc. In XX females, the primitive gonad then differentiates into an ovary. Sex-specific cDNA libraries have been prepared from female and male genital ridges isolated 12-13 dpc. A subtractive cloning strategy is being used to identify female-specific gene products involved in early organogenesis of the ovary.

Oogenesis: The expression of the zona pellucida, an ovary-specific extracellular matrix composed of three glycoproteins (ZP1, ZP2, ZP3), serves as a marker of oocyte growth and differentiation in the adult female. The mouse and human ZP2 and ZP3 genes, transcripts and proteins have been characterized. A 12bp DNA sequence 200 bp upstream of the start of transcription of all four genes is necessary and sufficient for zona promoter activation of reporter genes microinjected into growing mouse oocytes. Efforts are currently underway to clone ZAP-1, the putative transcription factor that binds to this DNA element. These studies will provide important molecular details of mechanisms involved in the coordinate, oocyte-specific expression of the zona genes. ZAP-1 will additionally provide an early marker of oocyte growth and differentiation.

Fertilization: The three zona proteins are secreted and form an extracellular matrix that mediates the relatively species-specific events of fertilization. Using microinjection techniques to degrade endogenous zona transcripts and to introduce synthetic zona mRNAs, we are assessing protein-protein interactions in the assemblage of the zona matrix. To investigate the molecular biology of sperm-egg interactions mediated by the zona, we have established a mouse *in vitro* fertilization assay. We are using recombinant DNA techniques to create mutant zona proteins and mouse/human chimeric zona proteins. These will be tested for their ability to competitively inhibit fertilization, enabling establishment of structure-function correlations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15508-04 LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Structure in Regulation of Mammalian Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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PROFESSIONAL

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2

2

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

The epsilon-globin gene is the first of the beta-like globin genes to be expressed during human development. Maximal epsilon-globin synthesis occurs in the large nucleated erythroid cells of the embryonic yolk sac. Transcription of the gene gradually ceases between the 6th and 10th weeks of fetal life, as the site of erythropoiesis shifts to the fetal liver. To investigate the regulation of this gene we have mapped, *in vitro*, the sites of interaction between nuclear proteins from erythroid and non-erythroid cells, and DNA sequences in the epsilon-globin promoter. We identified a site for the erythroid factor GATA-1 at position -165 in the ϵ -globin promoter. GATA-1 binding at this site is required to mediate the effect of the human beta-globin LCR HS II enhancer. However, in the absence of the enhancer GATA-1 does not participate in transcription from this promoter. GATA-1 sites in the enhancer could not replace the requirement for, nor did they interact with, the promoter site. The enhancer depended instead upon AP-1/NF-E2 sites in order to effect enhancement from this promoter. Thus, productive promoter-enhancer interactions increasing transcription of the ϵ -globin gene may require as few as two proteins interacting through two regulatory sites in the DNA.

The beta-globin LCR exhibits at least two kinds of properties: it has long range effects on chromatin structure, as well as classical enhancer activity. We have designed a minichromosomal vector containing a marked epsilon-globin gene, in order to study the effect of LCR sequences on the structure of the epsilon-globin gene in chromatin. The minichromosomes are carried as stable episomal elements, assembled into chromatin, in erythroid and non-erythroid human cells. In the absence of the LCR, we found that the epsilon-globin gene on the minichromosome was not transcribed. The gene may require its own enhancer to be expressed, even in an erythroid environment, suggesting that the availability of erythroid transcription factors is insufficient to allow expression. The minichromosome system may provide a means to study the effects of the LCR on chromatin structure, as well as its enhancer activity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 15509-01 LCDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of Polyprotein Processing in Retroviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

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2.0

PROFESSIONAL:

2.0

OTHER

0

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- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

Experiments have been initiated to study the chemical mechanism of the autoprocessing of the human immunodeficiency virus (HIV-1) protease (PR) from fusion proteins. An assay has been defined in which the wild-type fusion protein undergoes time-dependent autoprocessing concomitant with the appearance of HIV-1 PR activity. Analysis of the time course of the reaction monitored by immunoblotting showed the initial appearance of an intermediate product of cleavage of the fusion protein. This reaction was determined to be first-order from initial rate measurements of the appearance of enzymatic activity, and the disappearance of the full-length fusion protein. Pepstatin A inhibits autoprocessing with an inhibition constant that is at least fivefold lower than the inhibition constant for the mature PR. These results are consistent with a mechanism in which the processing event to release HIV-1 PR activity from the fusion protein involves intramolecular cleavage at the amino terminus from a dimer polyprotein. Inhibition of this reaction by pepstatin A and HIV-1 PR substrate indicates that the substrate binding site is intact in the full-length polyprotein.

The other approach involves structure to function characterization of HIV-1 polyprotein processing by mutational analysis. By characterizing the *in vitro* processed products of the purified full-length HIV-1 PR fusion proteins which contain mutations at either the N- or the C-terminal cleavage sites, we have identified mutations which completely block one of the cleavage sites of the PR and effect cleavage at the non-mutated wild-type site. In addition, we show that the sequence specificity for cleavage is more stringent at the N-terminus of the PR than at the C-terminus.

A rapid method has been described by which the PR can be expressed and purified to yield enzyme with high specific activity. A buffer system has been established for the stabilization of the PR that would allow kinetic and dimerization studies in solution. Production of the enzyme has aided in two detailed collaborative studies: (i) Studies on the effect of salt on the kinetic parameters of retroviral proteases in comparison with mammalian aspartic acid proteases; and (ii) Kinetic studies of the subsite specificity of HIV-1 and HIV-2 proteinases.

ANNUAL REPORT
OF
THE LABORATORY OF BIOCHEMISTRY AND METABOLISM
NATIONAL INSTITUTE OF DIABETES AND
DIGESTIVE AND KIDNEY DISEASES

The Laboratory conducts research in several apparently disparate areas that include morphogenesis, development, endocytosis, endocrinology, membrane transport, detoxication and the physical and chemical behavior of proteins and nucleic acids. It does so by applying a broad array of different approaches. Resolution is being attempted by methods that stem from biochemistry, biophysics, carbohydrate chemistry, cell biology, genetics and molecular biology. Although seemingly diverse, the common element to each of the research areas is appropriate to the Laboratories' designation: biochemical, metabolic and physical approaches are being brought to bear on major problems encompassed by the Institute's charge. It is the close proximity of experienced investigators from diverse scientific disciplines, able to discuss their individual problems with each other, that provides synergistic effects for resolution of the questions under investigation.

I. ENZYMES: FUNCTIONAL AND ABNORMAL

Several groups are active in this broadly designated area which covers the search for genes of lysosomal enzymes whose absence leads to disease; a genetic approach to the basic catalytic mechanism that is responsible for the sulfation of otherwise toxic substances; and the means by which enzymes are folded into the proper, active configuration.

Inherited Disorders of Lysosomal Function

N-acetylglucosaminyl-lysosomal enzyme-1-phosphotransferase is a membrane bound enzyme that is pivotal in equipping other enzymes with phosphorylated mannose moieties, residues which target lysosomal enzymes to lysosomes. Deficiency of this phosphotransferase results in two inherited disorders, Mucopolipidosis I and II. Isolation of the enzyme to obtain sequence data for use in cloning has been based on the selective affinity of the phosphotransferase for a conformationally dependent protein determinant shared by all lysosomal enzymes. Phosphotransferase activity in Hela cell membranes -- at least 70% of it -- will bind to a recombinant lysosomal enzyme, β -hexosaminidase B. This complex can be isolated with antibody to β -hexosaminidase B. Use of ^{35}S labeled membranes demonstrates that the affinity procedure provides a major purification step and suggests that a 67 kDa protein is a component of the phosphotransferase.

Another enzyme, N-acetyl-neuraminidase is deficient in the inherited lysosomal storage disorders, sialidosis and galactosialidosis. The former disease is thought to arise from mutations in the neuraminidase structural gene, whereas the latter

results from a defect in a protective glycoprotein which appears essential for maintenance of activity of both neuraminidase and β -galactosidase. All three proteins copurify and are believed to exist as a functional complex within the lysosome. Two approaches are being utilized to clone the mammalian neuraminidase. (1) The complex has been purified for use in isolation of neuraminidase to obtain sequence data for cloning. (2) A set of degenerate primers, based on a five amino acid sequence motif found in viral, bacterial and trypanosome neuraminidases, along with mRNA from human fibroblasts, has yielded several cDNA fragments following PCR amplification. Sequence analysis of the fragments will demonstrate whether an authentic part of human neuraminidase has been obtained for use in screening a cDNA library from bovine testicular tissue.

Enzymatic Basis of Detoxication

Previous work from this laboratory had delineated a number of sulfotransferases that were purified to homogenous form from mammalian livers and characterized. In this manner, phenol sulfotransferases of different types, alcohol sulfotransferases, and an amine susulfotransferase were identified and their mechanism of catalysis examined. All of these enzymes displayed the very broad specificity for lipophilic compounds that characterizes the enzymes of detoxication, a property that is useful in correlating physical and chemical properties of substrates with the protein's catalytic effectiveness.

Recently, a cDNA for tyrosine-ester sulfotransferase, has been obtained and is being expressed in large quantities by a bacterium. The resultant protein is entirely similar to the original mammalian species in catalytic capacity, although two separable protein species are obtained; the differences between the two may be due to differences in protein folding.

Attempts at obtaining site directed mutations have led to the isolation of about a dozen single-site mutants as well as of deletions. Several of the resultant mutant enzymes have been expressed, brought to homogeneity and tested for activity with a broad range of substrates. Although this work is entirely preliminary, it is apparent that quantitative changes in substrate utilization exist and that changes in secondary structure, measured by circular dichroism, have occurred as the result of mutations.

Protein Structure and Enzyme Mechanisms

Another laboratory is engaged in studies of protein structure and the mechanism by which a protein molecule, which is synthesized as a random coil, can fold into a specific secondary and tertiary structure, without any external help. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight 39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of its first 44 amino acids to produce an enzymatically active protein, pepsin. Pepsin is stable only at a pH below 6. Both proteins are unfolded by exposure to high pH, temperature or concentrations of such denaturants as urea. After unfolding, pepsinogen can refold to its normal

structure, when returned to native conditions, whereas pepsin cannot. Interest is in the mechanism of this refolding reaction and on the influence of the change in sequence on the behaviour of the two proteins. Using techniques such as ultra-violet, circular dichroic and fluorescence spectroscopies, together with chemical modification and peptide chemistry, the structures of the native and unfolded species have been characterized. Using rapid kinetic techniques, such as stopped-flow and T-jump, intermediate, partly folded forms have been detected in the folding reaction, their structures have been partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.

II. MORPHOGENESIS AND DEVELOPMENT

At a higher level of organization, one can question how enzymes or regulatory genes affect cell structures or the function of entire glands.

Polysaccharides in Morphogenesis

Chitin synthetases 1, 2 and 3 have been shown to have distinct functions in septum formation and cell separation in yeast. By carrying out several in-frame deletions, it has been established that an amino terminal portion of chitin synthetase 2 (Chs2) is not essential for activity or function. This is also probably true of the corresponding region of chitin synthetase 1 (Chs1). Experiments on the turnover of Chs1, Chs2 and Chs3 activity, and of the corresponding messenger RNA's after shutting off transcription, indicate that *in vivo* regulation of these enzymes probably occurs at the post-translation level.

Synthesis of the major structural polysaccharide of the yeast cell wall, $\beta(1\rightarrow3)$ glucan, is catalyzed by a membrane-bound system. By extraction with salts and detergents two fractions, A and B, have been solubilized that are needed, in combination with GTP, for glucan synthetase activity. Results with partially purified fraction A (the GTP-binding component) indicate that it may contain an intrinsic GTPase, whose modulation may be important for the control of both glucan synthetase activity and cell wall growth.

Tissue Specific and Hormone Regulated Gene Expression

The molecular basis of mammary-specific and developmentally and hormonally regulated gene expression was studied by analysis of the mouse whey acidic protein (WAP) gene in transgenic animals. Mammary specific transcription elements and control elements conferring hormone regulation are located in the promoter/upstream region, and probably also within the body of the gene. It was shown that chromatin surrounding WAP transgenes can modify the response of transcription elements to hormonal and developmental stimuli. Matrix attachment regions can insulate transcription elements from such position effects, thereby emphasizing the role of chromatin domains in regulated gene expression.

In continuation of establishing the mammary gland as a bioreactor, regulatory elements from the mouse WAP gene were found to be ideal for protein production in milk of transgenic swine and sheep. WAP regulatory elements are currently employed to produce human proteins such as tissue plasminogen activator, Protein C and glucocerebrosidase.

The precocious expression of WAP in mammary tissue during pregnancy can result in an abrogation of mammary development and a *milchlos* phenotype. Ectopic expression of WAP in the salivary gland of transgenic mice did not interfere with development of the gland, suggesting that WAP may be a mammary specific, developmental differentiation factor.

A technology was developed for introducing DNA into somatic tissues of live animals using jet injection. This method may be useful for generating somatic transgenics, for attempts at tumor cell ablation and for gene therapy.

III. PROTEIN SORTING AND TRANSPORT FUNCTIONS

Central to modern biology is the nature of the mechanism for the movement of macromolecules, glycoproteins in particular, not only into and out of the cell but also into specific organelles. The mechanisms involved are being sought from the approaches of the disciplines of somatic cell genetics, molecular biology, carbohydrate chemistry, endocrinology and biochemistry. The implications of the work extend from cell biology to applications in thyroid pathobiology and approaches to the AIDS virus.

Role of Carbohydrate Moiety of Glycoproteins

Work on the role of the carbohydrates of glycoproteins represents a continuation and extension of earlier studies on the role of protein-bound carbohydrates in biological systems. Three separate areas have been examined. (a) Current concepts of the mechanism of tritiated borohydride reduction of carbohydrates have been challenged and found wanting. Reduction under controlled conditions revealed the specific radioactivity of the individual sugars to vary widely and to be a function of the spatial environment of the carbonyl group being reduced. (b) Further understanding of the mechanism relating congenital goiter with hyposialylated thyroglobulin (described in an earlier report) has been attained by the demonstration in a rat thyroid cell line that TSH down-regulates mRNA for the enzyme, $\alpha 2,6$ sialyltransferase. The latter is responsible for the major portion of thyroglobulin sialyl residues which in turn affects iodination and hormone production. (c) An examination of the putative presence and potential role of carbohydrate-bound nuclear proteins has been initiated. Attention has been centered on those proteins which are tightly bound to DNA and which can be recovered from hydroxylapetite-bound chromatin. Preliminary findings point to the presence of small amounts of galactose and galactosamine.

Role of the Nuclear Envelope in Intracellular Protein Sorting

Transport across the nuclear pore complex is an essential process for regulating cell growth and normal development. Here, the structure of the nuclear pore and its involvement in nuclear transport are being studied at a molecular level. The laboratory had shown that the nuclear pore complex consists of a family of glycoproteins having covalently attached O-linked N-acetylglucosamine. Glycosylation of the major nuclear pore glycoprotein p62 has been examined in detail. After rat cDNA and gene encoding rat p62 were isolated, the primary sequence of p62 was found to consist of a series of 14 repeating pentapeptide motifs having the sequence GFSFG. Rabbit antisera raised against this pentapeptide react not only with p62 but also with the other members of the nuclear pore glycoprotein family. These antisera have been used to isolate the cDNAs encoding other nuclear pore glycoproteins. In addition, degenerate oligonucleotides, corresponding to the pentapeptide repeat, have been used as primers for isolating additional cDNA species by using the polymerase chain reaction technique. To examine the function of the nuclear pore glycoproteins in nuclear transport, an *in vitro* nuclear assembly and transport system was used. DNA, when added to extracts of *Xenopus laevis* nuclei, reform with intact nuclei envelopes and nuclear pores. These extracts can be depleted of endogenous nuclear pore components and reconstituted with recombinant or biochemically altered pore proteins. Using this assay, nuclear pore assembly and transport were shown to require nuclear pore glycoproteins; the O-linked N-acetylglucosamine moiety can be modified without altering transport. The ability to generate recombinant forms of other nuclear pore glycoproteins should allow a further dissection of the functional roles of these nuclear pore components.

Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

The characterization of iodide transport in thyroid is being continued. The current emphasis is to clone proteins in thyroid that are involved in the transport of iodide across the membrane. This project involves the use of rat thyroid cells in culture, and of cells unable to transport iodide; the latter cells were transfected with specific clones, enabling them to transport iodide.

Selection of the initial clones was based on the affinity of some proteins for binding to stilbene, since stilbenes interact with iodide transport. These clones were transfected into COS-7 cells and to non-iodide transporting thyroid cells. By these means transfected cells did not demonstrate properties of the major iodide uptake system of thyroid, but rather properties related to acid/base regulation and the maintenance of internal pH; the transfected cells show a pH-dependent loss of iodide. The effort to clone the major iodide-transport protein, therefore, required a different approach. The new methods depend on RNA-expression libraries and offer a promising path toward further understanding of iodide transport.

Collaborative studies show that thyroglobulin glycosylation regulated by TSH, plays a role in some forms of congenital goiter, and allows an understanding of critical aspects of thyroid hormone formation. Biochemical studies on thyroid

tissue from iodine-deficiency goiters relate iodine utilization, growth and thyroid hormone synthesis.

Cell Regulation by Hormones, Growth Factors, Autoantibodies, and Oncogenes

One group is studying the structure-function relationships of the thyrotropin (TSH) receptor, the involvement of this receptor in autoimmune thyroid disease, and the relationship between thyroid autoimmune diseases and other organ-specific autoimmune diseases, i.e. lupus and diabetes. Structure/ function of the TSH receptor are being compared to other glycoprotein hormone receptors and the regulation of thyroid function and growth by the TSH receptor is being evaluated and compared to receptors for other hormonal ligands. Particular attention is given to identifying determinants on the receptors important for TSH and receptor autoantibody binding and signal transduction, as well as the transcriptional and posttranscriptional mechanisms by which TSH and the other receptors affect thyroid gene expression. The relationship between oncogene transformation, the development of autoimmunity, and the loss of normal regulation of thyroid function and growth is applied to understanding why thyroid tumors and adenomas develop. The role of different signal transduction mechanisms - cAMP, Ca/ phosphoinositide and arachidonate - are being related to thyroid cell growth and differentiation, including thyroid hormone formation. Evaluation of the role of membrane lipids in regulation of TSH receptor expression, LDL receptor expression, and cholesterol biosynthesis is being undertaken as is a study of the role of major histocompatibility antigens in the development of autoimmune thyroid diseases and organ specific immune diseases. This work combines molecular biology, cell biology and monoclonal antibody methods in attacking the problem. A long term continuing project involves the development of thyroid cells which can grow in continuous culture in vitro and act as models of endocrine and thyroid disorders.

Endocytosis, Secretion and Compartmentalization

The analysis of complex cellular pathways by the approach of biochemical genetics forms the core of work of another group. The primary defect in FD1.3.25, a Chinese hamster ovary (CHO) cell mutant exhibiting genetically dominant aberrations in endocytosis and secretion, appears to reflect a single nucleotide substitution in one of the two alleles encoding the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase. The mutant polypeptide was originally identified on the basis of its persistent association with microtubule preparations, which has also been shown to be a genetically dominant trait; this correlates with the abnormal association of both late endosomes and secretory vesicles with microtubules observed in FD1.3.25. Studies directed toward elucidation of the interactions among microtubules, vesicles and the mutant polypeptide have been initiated.

Another strain, CHB11.1.3, isolated in this laboratory, like other CHO cell mutants utilizing polyprenol rather than dolichol in lipid-dependent N-linked glycosylation reactions, shows its greatest deficit with respect to synthesis of the nonamannosyl oligosaccharide. Recent results may prove significant in

uncovering the relationship between the structure of the lipid and the nature of the oligosaccharide synthesized. First, it has been shown that in CHB11.1.3, and in two independent dolichol mutants supplied by others, the synthesis of nonamannosyl oligosaccharide is restored by shifting the cells to 0°. A phenotypic revertant of CHB11.1.3, RR1.3.4, is the only one described to date that exhibits restoration of dolichol synthesis; RR1.3.4 accomplishes this through overproduction of polyprenol.

Intracellular Traffic in HIV Infection

Transport across the nuclear membrane is necessary at several steps in the life cycle of HIV. Once in the cytoplasm, the viral RNA is converted to double stranded DNA which must enter the nucleus. Viral regulatory proteins enter the nucleus while viral transcripts are exported into the cytoplasm. The viral REV protein has been identified as a key regulator of transport of HIV envelope mRNA. Several approaches have been taken to understand how REV may function. Cell lines are being prepared which express a REV/glucocorticoid receptor chimera that enters the nucleus in an inducible fashion; these cell lines also contain a construct containing a REV response element controlling expression of a chloramphenicol acetyltransferase (CAT) gene. The system allows CAT expression to be conveniently followed by direct measure of export of CAT mRNA into the cytoplasm. A means has also been devised for generating nuclei *in vitro* around exogenously added DNA; the method uses extracts from Xenopus laevis eggs. The nuclei assembled in such extracts mimic interphase nuclei in many ways and carry out active nuclear transport. These preparations allow examination of the mechanism of REV action and the movement of other molecules involved in the HIV life cycle. In other studies, the structure of the nuclear pore has been examined. The nuclear pore requires glycoprotein components for proper morphology and function. Since previous studies indicate that overexpression of nuclear pore glycoproteins may be toxic to cells, the cDNA encoding the major nuclear glycoprotein p62 has been placed under the control of a glucocorticoid responsive expression vector. By expressing the sense and antisense constructs, it should be possible to study the effects of suppression or overexpression of this nuclear pore component. A number of the components of the pore complex have now been molecularly cloned. Understanding how retroviral products cross the nuclear envelope is critical to attempts to regulate or inhibit the critical steps in the HIV life cycle.

IV. HYDRATION FORCES

The more physical interactions of macromolecules -- particularly proteins, lipids and DNA -- with their environment is being studied with special focus on the role of water.

Direct Measurement of Forces between Membranes or Macromolecules

The ability to measure directly the forces between membranes or between macromolecules is creating a new logic for thinking about molecular recognition, assembly, and folding. The outstanding feature of interaction is that as molecules

or membranes approach contact, the important work of approach involves removal of organized water solvent from the apposing surfaces. These "hydration forces" are now recognized to act in materials as diverse as lipid bilayers, proteins, DNA double helices, and stiff polysaccharides.

The first direct measurement of forces between protein molecules (type I collagen triple helices) has now been performed and shown to have all the features characteristic of hydration forces. The temperature dependence of the force is similar to that observed in ordered arrays of DNA molecules, i.e. the physical nature of temperature-favored assembly in DNA and proteins may be similar. Temperature-favored assembly is a common feature of many biologically important processes. A theory of temperature-favored assembly induced by attractive hydration forces between hydrophilic molecules has been developed. Measurement of interaction forces between dihexadecyldimethylammonium acetate bilayers has demonstrated that neither thermal-mechanical undulations nor molecular protrusions contribute significantly to hydration forces between lipid bilayers. A dependence of hydration forces between DNA molecules on solution ion composition has been studied. An unusual H₂O-L-H₂O double phase transition induced by variation of water concentration in lipid/water mixtures has been observed and explained. A theoretical model relating hydration forces and phase transitions on the surfaces of interacting molecules has been suggested.

Physics of Ionic Channels and other Proteins with Aqueous Cavities

Through the use of osmotic stress, the regulatory effect of chloride ions on hemoglobin function has been reexamined, explicitly including water effects. Instead of the 1.6 chloride ions generally assumed linked to oxygen affinity, it now appears that the direct binding of only one ion to the deoxy state is linked, while the rest of the effect is due to the binding of 65 extra water molecules to the proteins in the oxy state. Taking into account hydration effects, this result suggests that ligand regulation of "allosteric proteins" must be re-evaluated with new regard for the activity of water.

The peptide alamethicin, inserted into bilayer membranes composed of lipids known to form inverted phases of different spontaneous radii, showed a clear correlation between the lipid spontaneous curvature and the relative probabilities of different conductance states and, therefore, channel structures. The dependence of ionic channel expression on the packing strain in lipid bilayer revealed by these experiments suggests an active role for membrane lipid composition in regulating membrane protein activity.

Currents through fully open single channels formed by *Staphylococcus aureus* alpha toxin were subjected to fluctuation analysis. A new mechanism of excess noise generation in an ion channel was identified and related to a reversible ionization of residues in the channel-forming molecule. The reaction parameters and the number of residues participating in the ionization process were extracted. The ability of noise analysis to study reactions within a single ionic channel demonstrates the potential power of the technique as a structural tool for channel function analysis.

Structure and Physical Properties of DNA and DNA-Protein Complexes

The effect of small, neutral molecules on the binding of sequence specific proteins to DNA is being studied both to understand the energetics underlying this important class of molecular recognition reactions and to develop a method for stabilizing the DNA-protein complexes of activated gene complexes for structural studies using transient electric birefringence and dichroism.

The fundamental interaction of sugars with DNA and proteins has been probed through their influence on forces measured by the osmotic stress method coupled with x-ray diffraction. Sugars are preferentially included in the DNA phase. Interaction coefficients scale linearly with sugar molecular weight. These direct force measurements can be connected with experiment. Both the B-Z transition of poly(dG-dC) and the molten globule - random coil transition of myoglobin show osmotic sensitivities that also scale with sugar molecular weight.

The osmotic sensitivity of the galactose operon repressor, binding to its operator sequences, is being measured. The binding of two repressors at sites separated by about 100 base pairs is necessary for function. The binding of the first is accompanied by the uptake of some 25 extra water molecules; the binding of the second shows the release of about 200 waters. The binding of the first appears to trigger a protein conformational change exposing extra surface area; while the binding of the second releases this extra bound water through loop formation mediated by protein interactions.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 17001-26 LBM

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of Carbohydrate Moiety of Glycoproteins in Cellular Activity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G. Ashwell Institute Scholar LBM, NIDDK

Others: W. Berlin IRTA LBM, NIDDK
O. Gabriel Research Chemist LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Biochemistry and Metabolism

SECTION Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.5

PROFESSIONAL: 2.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The work carried out this year represents a continuation and extension of earlier studies on the role of protein-bound carbohydrates in biological systems. Three separate areas have been examined. (a) Current concepts of the mechanism of tritiated borohydride reduction of carbohydrates have been challenged and found wanting. Reduction under controlled conditions revealed the specific radioactivity of the individual sugars to vary widely and to be a function of the spatial environment of the carbonyl group being reduced. (b) Further understanding of the mechanism relating congenital goiter with hyposialylated thyroglobulin (described in an earlier report) has been attained by the demonstration in a rat thyroid cell line that TSH down-regulates mRNA for the enzyme, α 2,6 sialyltransferase. The latter is responsible for the major portion of thyroglobulin sialyl residues which in turn affects iodination and hormone production. (c) An examination of the putative presence and potential role of carbohydrate-bound nuclear proteins has been initiated. Attention has been centered on those proteins which are tightly bound to DNA and which can be recovered from hydroxylapatite-bound chromatin. Preliminary findings point to the presence of small amounts of galactose and galactosamine.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 17002-22 LBM

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Enzymatic Basis of Detoxication

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.B. Jakoby Chief, LBM LBM, NIDDK

Others: Y-S. Yang Visiting Associate LBM, NIDDK
X. Chen Visiting Fellow LBM, NIDDK
A. Guo Visiting Fellow LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Biochemistry and Metabolism

SECTION Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 3.5

PROFESSIONAL: 3.0

OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous work from this laboratory had delineated a number of sulfotransferases that had been purified to homogenous form from mammalian livers and characterized. In this manner, phenol sulfotransferases of different types, alcohol sulfotransferases, and an amine susulfotranferase were identified and their mechanism of catalysis examined. All of these enzymes displayed the very broad specificity for lipophilic compounds that characterizes the enzymes of detoxication, a property that is useful in correlating physical and chemical properties of substrates with the protein's catalytic effectiveness.

Recently, a cDNA for tyrosine-ester sulfotransferase, has been obtained and is being expressed in large quantities by a bacterium. The resultant protein is entirely similar to the original mammalian species in catalytic capacity, although two separable protein species are obtained; no difference between the two may be due to differences in folding.

Attempts at obtaining site directed mutations have led to the isolation of about a dozen single-site mutants as well as of deletions. Several of the resultant mutant enzymes have been expressed, brought to homogeneity and tested for activity with a broad range of substrates. Although this work is entirely preliminary, it is apparent that quantitative changes in substrate utilization exist and that changes in secondary structure, measured by circular dichroism, have occurred as the result of mutations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 17003-25 LBM
PERIOD COVERED October 1, 1991 through September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Polysaccharides in Morphogenesis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> PI: E. Cabib </div> <div style="width: 30%;"> Senior Research Chemist </div> <div style="width: 30%;"> LBM, NIDDK </div> </div> <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> Others: P.C. Mol J.A. Shaw W.-J. Choi Roman Kollar Emanuela Lacanà </div> <div style="width: 30%;"> Visiting Fellow Staff Fellow Visiting Fellow Special Volunteer Visiting Fellow </div> <div style="width: 30%;"> LBM, NIDDK LBM, NIDDK LBM, NIDDK LBM, NIDDK LBM, NIDDK </div> </div>		
COOPERATING UNITS (If any) LCB, NHLBI (Blair Bowers); Departamento de Microbiologia, University of Salamanca, Spain (Angel Duran); Slovak Academy of Sciences, Institute of Chemistry, Bratislava, Czechoslovakia (Vladimir Farkas); CRADA, American Cyanamid, Princeton, NJ		
LAB/BRANCH Laboratory of Biochemistry and Metabolism		
SECTION Section on Morphogenesis		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS 5.5	PROFESSIONAL 5.5	OTHER
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div style="width: 30%;"> <input type="checkbox"/> (b) Human tissues </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Previous work has shown that chitin synthetases 1, 2 and 3 have distinct functions in septum formation and cell separation in yeast. By carrying out several in-frame deletions, it has been established that an amino terminal portion of chitin synthetase 2 (Chs2) is not essential for activity or function. This is also probably true of the corresponding region of chitin synthetase 1 (Chs1). Experiments on the turnover of Chs1, Chs2 and Chs3 activity and of the corresponding messenger RNA's after shutting off transcription indicate that <u>in vivo</u> regulation of these enzymes probably occurs at the post-translation level.</p> <p>Synthesis of the major structural polysaccharide of the yeast cell wall, $\beta(1\rightarrow3)$ glucan, is catalyzed by a membrane-bound system. By extraction with salts and detergents two fractions, A and B, have been solubilized that are needed, in combination with GTP, for glucan synthetase activity. Results with partially purified fraction A (the GTP-binding component) indicate that it may contain an intrinsic GTPase, whose modulation may be important for the control of glucan synthetase activity and of cell wall growth.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 17004-24 LBM

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thermodynamic and Kinetic Studies of Protein Structure and Enzymatic Mechanisms

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. McPhie Research Chemist LBM, NIDDK

Others:

COOPERATING UNITS (if any)

LMC, NHLI (Robert Adelstein); LCP, NIDDK (Edith Miles); MB, NCI (Jane Cheng); LPS, DCRT (Richard Shrager)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 1

PROFESSIONAL: 1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This laboratory is engaged in studies on protein structure and the mechanism by which a protein molecule, which is synthesized as a random coil, can fold into a specific secondary and tertiary structure, without any external help. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight=39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of it's first 44 amino acids, to produce an enzymatically active protein, pepsin. Pepsin is stable only at pH's below 6. Both proteins are unfolded by exposure to high pH, temperature or concentrations of denaturants, such as urea. After such unfolding, pepsinogen can refold to its normal structure, when returned to native conditions, whereas pepsin cannot. I am interested in the mechanism of this refolding reaction and on the influence of the change in sequence on the behaviour of the two proteins. Using techniques such as ultra-violet, circular dichroic and fluorescence spectroscopies, together with chemical modification and peptide chemistry, the structures of the native and unfolded species have been characterized. Using rapid kinetic techniques, such as stopped-flow and T-jump, intermediate, partly folded forms have been detected in the folding reaction, their structures have been partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 17008-09 LBM
PERIOD COVERED October 1, 1991 through September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <p style="text-align: center;">The Role of the Nuclear Envelope in Intracellular Protein Sorting</p>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	J.A. Hanover	Research Chemist LBM, NIDDK
Others:	M. Baker S. Bailer W. Lubas	Expert LBM, NIDDK Guest Researcher LBM, NIDDK Research Associate LBM, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Biochemistry and Metabolism		
SECTION Section on Cellular Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS 3.5	PROFESSIONAL 3.5	OTHER
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Transport across the nuclear pore complex is an essential process for regulating cell growth and normal development. The structure of the nuclear pore and its involvement in nuclear transport are being studied at a molecular level. Previously the laboratory has shown that the nuclear pore complex is made up of a family of glycoproteins having covalently attached O-linked N-acetylglucosamine. Glycosylation of the major nuclear pore glycoprotein p62 has been examined in detail. The rat cDNA and gene encoding rat p62 have been isolated. Examination of the primary sequence of p62 revealed a series of 14 repeating pentapeptide motifs having the sequence GFSFG. Rabbit antisera raised against this pentapeptide sequence react not only with p62 but also with the other members of the nuclear pore glycoprotein family. These antisera have been used to isolate the cDNAs encoding other nuclear pore glycoproteins. In addition, degenerate oligonucleotides corresponding to the pentapeptide repeat have been used as primers for isolating additional cDNA species using the polymerase chain reaction technique. To examine the function of the nuclear pore glycoproteins in nuclear transport, an <i>in vitro</i> nuclear assembly and transport system has been employed. When DNA is added to extracts of <i>Xenopus laevis</i> nuclei reform with intact nuclei envelopes and nuclear pores. These extracts can be depleted of endogenous nuclear pore components and reconstituted with recombinant or biochemically altered pore proteins. Using this assay nuclear pore assembly and transport were shown to require nuclear pore glycoproteins; the O-linked N-acetylglucosamine moiety can be modified without altering transport. The ability to generate recombinant forms of other nuclear pore glycoproteins should allow a further dissection of the functional roles of these nuclear pore components. </p>		

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17009-07 LBM

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Tissue Specific and Hormone Regulated Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: L. Hennighausen Research Chemist LBM, NIDDK

Others: M. Baik Visiting Fellow LBM, NIDDK
 R.A. McKnight Staff Fellow LBM, NIDDK
 A. Shamay Visiting Fellow LBM, NIDDK

COOPERATING UNITS (if any)

US Department of Agriculture (R. Wall, V. Pursel); NCI (G. Smith); NCI, Univ. of Maryland (P.A. Furth); NIMH (E. Ginns)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Developmental Biology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

3.5

PROFESSIONAL

3.5

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The molecular basis of mammary-specific and developmentally and hormonally regulated gene expression is being studied through the analysis of the mouse whey acidic protein (WAP) gene in transgenic animals. Mammary specific transcription elements and control elements conferring hormone regulation are located in the promoter/upstream region, and probably also within the body of the gene. It was shown that chromatin surrounding WAP transgenes can modify the response of transcription elements to hormonal and developmental stimuli. Matrix attachment regions can insulate transcription elements from such position effects, thereby emphasizing the role of chromatin domains in regulated gene expression.

In continuation of establishing the mammary gland as a bioreactor, it was shown that regulatory elements from the mouse WAP gene are ideal for protein production in milk of transgenic swine and sheep. WAP regulatory elements are currently employed to produce human proteins such as tissue plasminogen activator, Protein C and glucocerebrosidase.

It has been shown that precocious expression of WAP in mammary tissue during pregnancy can result in an abrogated mammary development and a *milchlos* phenotype. Ectopic expression of WAP in the salivary gland of transgenic mice did not interfere with development of the gland, suggesting that WAP may be a mammary specific developmental/differentiation factor.

A technology was developed to introduce DNA into somatic tissues of live animals using jet injection. This technology may be useful for generating somatic transgenics, for tumor cell ablation and for gene therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17024-09 LBM

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Inherited Disorders of Lysosomal Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. Myerowitz	Research Chemist	LBM, NIDDK
Others:	J.A. Boose	Staff Fellow	LBM, NIDDK
	A. Conley	Biologist	LBM, NIDDK
	J. Tropea	Staff Fellow	LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Inherited Metabolic Disease

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.3

PROFESSIONAL

1.4

OTHER

.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

N-acetylglucosaminyl-lysosomal enzyme-1-phosphotransferase is a membrane bound enzyme that is pivotal in equipping lysosomal enzymes with phosphorylated mannose moieties, residues which target lysosomal enzymes to lysosomes. Deficiency of the enzyme results in the inherited disorders Mucopolipidosis I and II. Isolation of the enzyme to obtain sequence data for use in cloning has been based on the selective affinity of the phosphotransferase for a conformationally dependent protein determinant shared by all lysosomal enzymes. 70% of the phosphotransferase activity in Hela cell membranes will bind in solution to a recombinant lysosomal enzyme, β -hexosaminidase B, and that this complex can be isolated with antibody to β -hexosaminidase B. Use of ^{35}S labeled membranes demonstrates that the affinity procedure provides a major purification step and suggests that a 67 kDa protein is a component of the phosphotransferase. The feasibility of using the Vaccinia/T7 transient expression system for cDNA cloning of the phosphotransferase has been examined.

N-acetyl-neuraminidase is deficient in the inherited lysosomal storage disorders, sialidosis and galactosialidosis. The former disease is thought to arise from mutations in the neuraminidase structural gene, whereas the latter results from a defect in a glycoprotein (protective protein) which appears essential for maintenance of activity of both neuraminidase and β -galactosidase. All three proteins copurify and are believed to exist as a functional complex within the lysosome. Two approaches are being utilized to clone the mammalian neuraminidase. (1) The complex has been purified from bovine testicular tissue for use in isolation of neuraminidase to obtain sequence data for cloning. (2) A set of degenerate primers, based on a five amino acid sequence motif found in viral, bacterial and trypanosome neuraminidases, along with mRNA from human fibroblasts has yielded several cDNA fragments following PCR amplification. Sequence analysis of the fragments will demonstrate whether an authentic part of human neuraminidase has been obtained for use in screening a cDNA library.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18007-13 LBM

PERIOD COVERED
October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: E.F. Grollman Medical Officer (Research) LBM, NIDDK

Others: M.C. Baggio Visiting Fellow LBM, NIDDK
Y. Shimura Guest Worker LBM, NIDDK

COOPERATING UNITS (if any)

LBM, NIDDK (L.D. Kohn, M. Saji, G. Ashwell); Walter Reed (S. Aloj); Roswell Park Cancer Inst. (J.YT Lau); Univ. Sao Paulo (Medeiros-Neto).

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Cell Regulation

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 10892

TOTAL MAN-YEARS

2.25

PROFESSIONAL

2.0

OTHER

0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The present project continues to address the characterization of iodide transport in thyroid. The current emphasis is to clone proteins in thyroid that are involved in the transport of iodide across the membrane. This project involves the use of rat thyroid cells in culture, and the use of cells that are unable to transport iodide; these cells are transfected with specific clones enabling them to transport iodide.

Selection of the initial clones was based on the property of stilbene binding, since stilbenes interact with iodide transport. These clones were transfected into COS-7 cells and to non-iodide transporting thyroid cells. The results using these methods show that the transfected cells do not demonstrate the major iodide uptake system of thyroid, but rather the property involved relates to acid/base regulation and maintenance of internal pH. The transfected cells have characteristics of pH-dependent loss of iodide. The effort to clone the major iodide-transport protein has led to the use of a different approach. These new methods depend on RNA-expression libraries. Initial studies are complex and time-consuming but offer a promising approach to further understand iodide transport. Furthermore, in co-ordination with Dr. Ashwell, this laboratory is completing work involving the glycosylation of thyroglobulin, the major secretory product of thyroid. These studies show that thyroglobulin glycosylation is TSH-regulated, plays a role in some forms of congenital goiter, and finally is essential to understand critical aspects of thyroid hormone formation. Finally, biochemical studies on thyroid tissue from iodine-deficiency goiters relate iodine utilization, growth and thyroid hormone synthesis.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18008-26 LBM

PERIOD COVERED: 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders):
Cell Regulation by Hormones, Growth Factors, Autoantibodies, and Oncogenes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L.D. Kohn, Medical Director, USPHS, and Chief, Sec on Cell Regulation	LBM, NIDDK
Others:	T. Ban	Visiting Fellow LBM, NIDDK
	H. Shimura	Visiting Fellow LBM, NIDDK
	S. Kosugi	Visiting Fellow LBM, NIDDK
	A. Hidaka	Visiting Fellow LBM, NIDDK
	M. Saji	Special Volunteer (1 yr) LBM, NIDDK
	H. Niller	Special Volunteer (3 mos) LBM, NIDDK
	S. Ikuyama	Special Volunteer (5 mos) LBM, NIDDK
	F. Okajima	Special Volunteer (5 mo.) LBM, NIDDK

COOPERATING UNITS (if any)

NIDDK (E.F. Grollman); U. Pisa, Italy (A. Pinchera); U. Naples (R. DiLauro, E. Avvedimento, S. Aloj, & E. Consiglio); NCI (D. Singer); U. Texas Galveston (B. Prabhakar); Baylor U., Houston (F. Ledley); U. MD (W.A. Valente); Tokyo Women's U, Tokyo, Japan (O. Isozaki); U. of Kyoto, Kyoto, Japan (T. Mori); French CNR, Paris, France (J. Charrier).

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Cell Regulation

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

8.1

PROFESSIONAL

7.1

OTHER

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This laboratory is studying the structure-function relationships of the thyrotropin (TSH) receptor, the involvement of this receptor in autoimmune thyroid disease, as well as the relationship between thyroid autoimmune diseases and other organ-specific autoimmune diseases, i.e. Lupus or diabetes. We compare structure/function of the TSH receptor to other glycoprotein hormone receptors and evaluate the interdependent regulation of thyroid function and growth by the TSH receptor and receptors for other ligands: gonadotropins, adrenergic, cholinergic, insulin, insulin-like growth factors (I and II), fibroblast growth factors, hydrocortisone, thyroid hormones, purinergic, bacterial toxins (cholera, pertussis, tetanus), interferon, and interleukins. Particular attention is given to identifying determinants on the receptors important for TSH and receptor autoantibody binding and signal transduction, as well as the transcriptional and posttranscriptional mechanisms by which TSH and the other receptors affect thyroid gene expression. The relationship between oncogene transformation, the development of autoimmunity, and the loss of normal regulation of thyroid function and growth is applied to understanding why thyroid tumors and adenomas develop. We relate the role of different signal transduction mechanisms - cAMP, Ca²⁺/phosphoinositide and arachidonate - to thyroid cell growth and differentiation, including thyroid hormone formation. We evaluate the role of membrane lipids in regulation of TSH receptor expression, LDL receptor expression, and cholesterol biosynthesis. We study the role of major histocompatibility antigens in the development of autoimmune thyroid diseases and organ specific immune diseases. These studies combine a molecular biology, cell biology and monoclonal antibody approach. A long term continuing project involves the development of thyroid cells which can grow in continuous culture in vitro and act as models of endocrine and thyroid disorders.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 18009-13 LBM

PERIOD COVERED
October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)
Endocytosis, Secretion and Compartmentalization in Mutant CHO Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. R. Robbins	Research Geneticist	LBM, NIDDK
Others:	C. W. Hall	Research Chemist	LBM, NIDDK
	T. M. Weber	Special Volunteer	LBM, NIDDK

COOPERATING UNITS (if any) MDB, NIDDK, NIH (Dr. Karen MacKay); LI, NIDR, NIH (Dr. Constance Oliver) Dept. of Biochemistry, School of Public Health and Hygiene, Johns Hopkins University (Prof Sharon S. Krag)

LAB/BRANCH Laboratory of Biochemistry and Metabolism

SECTION Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	3.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The research of this laboratory involves analysis of complex cellular pathways through biochemical genetics.

The primary defect in FD1.3.25, a Chinese hamster ovary (CHO) cell mutant exhibiting genetically dominant aberrations in endocytosis and secretion, appears to reflect a single nucleotide substitution in one of the two alleles encoding the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase. The mutant polypeptide was originally identified on the basis of its persistent association with microtubule preparations, also shown to be a genetically dominant trait; this correlates with the abnormal association of both late endosomes and secretory vesicles with microtubules observed in FD1.3.25. Studies directed toward elucidation of the interactions among microtubules, vesicles and the mutant polypeptide have been initiated.

CHB11.1.3, isolated in this laboratory, like other CHO cell mutants utilizing poly-prenol rather than dolichol in lipid-dependent N-linked glycosylation reactions, shows its greatest deficit with respect to synthesis of the nonamannosyl oligosaccharide. Recent results may prove significant in uncovering the relationship between the structure of the lipid and the nature of the oligosaccharide synthesized. First, it has been shown in CHB11.1.3 and two independent dolichol mutants supplied by others, that the synthesis of nonamannosyl oligosaccharide is restored by shifting the cells to 0°. Second, a phenotypic revertant of CHB11.1.3, RR1.3.4, is the only one described to date exhibiting restoration of dolichol synthesis; RR1.3.4 accomplishes this through overproduction of poly-prenol.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 18010-05 LBM
PERIOD COVERED October 1, 1991 through September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Role of Intracellular Traffic in HIV Infection		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	J.A. Hanover	Research Chemist
		LBM, NIDDK
Others:	M. Miller	IRTA
		LBM, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Biochemistry and Metabolism		
SECTION Section on Cellular Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1	1	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.) <p> Transport across the nuclear membrane is necessary at several steps in the life cycle of HIV. Once in the cytoplasm, the viral RNA is converted to double stranded DNA which must enter the nucleus. Viral regulatory proteins enter the nucleus while viral transcripts are exported into the cytoplasm. The viral REV protein has been identified as a key regulator of transport of HIV envelope mRNA. Several approaches have been taken to understand how REV may function. Cell lines are being prepared which express a REV/glucocorticoid receptor chimera which enters the nucleus in an inducible fashion; these cell lines also contain a construct containing a REV response element controlling expression of a chloramphenicolacetyltransferase (CAT) gene. This will allow CAT expression to be conveniently followed allowing a direct measure of export of CAT mRNA into the cytoplasm. A means has also been devised for generating nuclei <i>in vitro</i> around exogenously added DNA. The method uses extracts from <i>Xenopus laevis</i> eggs. The nuclei assembled in such extracts mimic interphase nuclei in many ways and carry out active nuclear transport. These preparations allow examination of the mechanism of REV action and the movement of other molecules involved in the HIV life cycle. In other studies, the structure of the nuclear pore has been examined. The nuclear pore requires glycoprotein components for proper morphology and function. Previous studies indicate that overexpression of nuclear pore glycoproteins may be toxic to cells. To circumvent these problems, the cDNA encoding the major nuclear glycoprotein p62 has been placed under the control of a glucocorticoid responsive expression vector. By expressing the sense and antisense constructs, it should be possible to study the effects of suppression or overexpression of this nuclear pore component. A number of the components of the pore complex have now been molecularly cloned. Understanding how retroviral products cross the nuclear envelope is critical to attempts to regulate or inhibit the critical steps in the HIV life cycle. </p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18011-03 LBM

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Cell Specific Activity of Elements within the HIV-LTR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory and institute affiliation)

PI: L. Hennighausen Research Chemist LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

This project was terminated as of September 30, 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 18012-08 LBM
PERIOD COVERED October 1, 1991 through September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Direct Measurement of Forces between Membranes or Macromolecules		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	V.A. Parsegian D.C. Rau	Guest Researcher Research Chemist LBM, NIDDK LBM, NIDDK
Others:	S. Leikin	Guest Researcher LBM, NIDDK
COOPERATING UNITS (if any) Brock University, Ontario, Canada (Dr. R.P. Rand); University of British Columbia, Vancouver, Canada (Dr. E.A. Evans); University of Minnesota, Minneapolis, MN (Dr. D.F. Evans); NHLBI (Dr. K. Gawrisch); Josef Stefan Institute, Ljubljana, Slovenia (Dr. R. Podgornik); Technical University of Munich, Munich, Germany (Dr. A.A. Kornyshev)		
LAB/BRANCH <div style="text-align: center;">Laboratory of Biochemistry and Metabolism</div>		
SECTION <div style="text-align: center;">Enzymes and Cellular Biochemistry Section/Biophysics Unit</div>		
INSTITUTE AND LOCATION <div style="text-align: center;">NIDDK, NIH, Bethesda, Maryland 20892</div>		
TOTAL MAN-YEARS	2.0	PROFESSIONAL <div style="text-align: center;">2.0</div>
		OTHER <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.) <p>The ability to measure directly the forces between membranes or between macromolecules is creating a new logic for thinking about molecular recognition, assembly, and folding. The outstanding feature of interaction is that as molecules or membranes approach contact, the important work of approach involves removal of organized water solvent from the apposing surfaces. These "hydration forces" are now recognized to act in materials as diverse as lipid bilayers, proteins, DNA double helices, and stiff polysaccharides.</p> <p>During the current year a first direct measurement of forces between protein molecules (type I collagen triple helices) has been done. It has been shown that the force has all the features characteristic of hydration forces. The temperature dependence of the force is similar to that observed in ordered arrays of DNA molecules. This shows that physical nature of temperature-favored assembly in DNA and proteins might be similar. Temperature-favored assembly is a common feature of many biologically important processes.</p> <p>A theory of temperature-favored assembly induced by attractive hydration forces between hydrophilic molecules has been developed. Measurement of interaction forces between dihexadecyldimethylammonium acetate bilayers has demonstrated that neither thermal-mechanical undulations nor molecular protrusions contribute significantly to hydration forces between lipid bilayers.</p> <p>A dependence of hydration forces between DNA molecules on solution ion composition has been studied.</p> <p>An unusual H-L-H double phase transition induced by variation of water concentration in lipid/water mixtures has been observed and explained.</p> <p>A theoretical model relating hydration forces and phase transitions on the surfaces of interacting molecules has been suggested.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 18013-05 LBM																								
PERIOD COVERED October 1, 1991 through September 30, 1992																										
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders) Physics of Ionic Channels and other Proteins with Aqueous Cavities																										
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">V.A. Parsegian</td> <td style="width: 40%;">Guest Researcher</td> <td style="width: 10%;">LBM, NIDDK</td> </tr> <tr> <td>Others:</td> <td>S. M. Bezrukov</td> <td>Visiting Scientist</td> <td>LBM, NIDDK</td> </tr> <tr> <td></td> <td>M.F. Colombo</td> <td>Visiting Fellow</td> <td>LBM, NIDDK</td> </tr> <tr> <td></td> <td>M.J. Curran</td> <td>Special Volunteer</td> <td>LBM, NIDDK</td> </tr> <tr> <td></td> <td>J.J. Kasianowicz</td> <td>Guest Researcher</td> <td>LBM, NIDDK</td> </tr> <tr> <td></td> <td>D.C. Rau</td> <td>Research Chemist</td> <td>LBM, NIDDK</td> </tr> </table>			PI:	V.A. Parsegian	Guest Researcher	LBM, NIDDK	Others:	S. M. Bezrukov	Visiting Scientist	LBM, NIDDK		M.F. Colombo	Visiting Fellow	LBM, NIDDK		M.J. Curran	Special Volunteer	LBM, NIDDK		J.J. Kasianowicz	Guest Researcher	LBM, NIDDK		D.C. Rau	Research Chemist	LBM, NIDDK
PI:	V.A. Parsegian	Guest Researcher	LBM, NIDDK																							
Others:	S. M. Bezrukov	Visiting Scientist	LBM, NIDDK																							
	M.F. Colombo	Visiting Fellow	LBM, NIDDK																							
	M.J. Curran	Special Volunteer	LBM, NIDDK																							
	J.J. Kasianowicz	Guest Researcher	LBM, NIDDK																							
	D.C. Rau	Research Chemist	LBM, NIDDK																							
COOPERATING UNITS (if any) LPTB, NICHD (Dr. J.J. Zimmerberg); John Hopkins Univ., Baltimore, MD (Dr. A. Harris); Office of Naval Research (Dr. I. Vodyanoy); St. George's Hospital, Univ. of London, England (Drs. C. Pasternak and L. Bashford); Worcester Foundation for Experimental Biology, Shrewsbury, MA (Dr. H. Bayley); Princeton Univ., Princeton, NJ (Drs. S.M. Gruner, M.W. Tate, S.L. Keller)																										
LAB/BRANCH Laboratory of Biochemistry and Metabolism																										
SECTION Enzymes and Cellular Biochemistry Section/Biophysics Unit																										
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892																										
TOTAL MAN-YEARS	PROFESSIONAL	OTHER																								
3.75	3.75																									
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																										
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Through the use of osmotic stress, the regulatory effect of chloride ions on hemoglobin function has been reexamined explicitly including water effects. Instead of the 1.6 chloride ions generally assumed linked to oxygen affinity, it now appears that the direct binding of only one ion to the deoxy state is linked, while the rest of the effect is due to the binding of 65 extra water molecules to the oxy state. This result taking into account hydration effects suggests that ligand regulation of "allosteric proteins" must be re-evaluated with new regard for the activity of water.</p> <p>The peptide alamethicin inserted into bilayer membranes composed of lipids known to form inverted phases of different spontaneous radii showed a clear correlation between the lipid spontaneous curvature and the relative probabilities of different conductance states and therefore channel structures. The dependence of ionic channel expression on the packing strain in lipid bilayer revealed by these experiments suggests an active role for membrane lipid composition in regulating membrane protein activity.</p> <p>Currents through fully open single channels formed by <i>Staphylococcus aureus</i> alpha toxin were subjected to fluctuation analysis. A new mechanism of excess noise generation in an ion channel was identified and related to a reversible ionization of residues in the channel-forming molecule. The reaction parameters and the number of residues participating in the ionization process were extracted. The ability of noise analysis to study reactions within a single ionic channel demonstrates the potential power of the technique as structural tool for channel function analysis.</p>																										

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 18014-08 LBM

PERIOD COVERED
October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Structure and Physical Properties of DNA and DNA-Protein Complexes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)
PI: D.C. Rau Research Chemist LBM, NIDDK

Others: M. Colombo Visiting Fellow LBM, NIDDK
N. Sidorova Visiting Fellow LBM, NIDDK

COOPERATING UNITS (If any)
LPTB, NICHD (Dr. M. Garner); George Mason University, Fairfax, VA (Dr. H.-H. Chen);
University of Nevada, Reno, Nevada (Dr. R.H. Harrington); University of Calgary, Alberta,
Canada (Dr. D. Bazett-Jones)

LAB/BRANCH Laboratory of Biochemistry and Metabolism

SECTION Enzymes and Cellular Biochemistry Section/Biophysics Unit

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
.5	.5	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The effect of small, neutral molecules on the binding of sequence specific proteins to DNA is being studied both to understand the energetics underlying this important class of molecular recognition reactions and to develop a method for stabilizing the DNA-protein complexes of activated gene complexes for structural studies using transient electric birefringence and dichroism.

The fundamental interaction of sugars with DNA and proteins has been probed through their influence on forces measured by the osmotic stress method coupled with x-ray diffraction. Sugars are preferentially included in the DNA phase. Interaction coefficients scale linearly with sugar molecular weight. These direct force measurements can be connected with experiment. Both the B-Z transition of poly (dG-dC) and the molten globule - random coil transition of myoglobin show osmotic sensitivities that also scale with sugar molecular weight.

The osmotic sensitivity of the E. coli galactose operon repressor binding to its operator sequences is being measured. The binding of 2 repressors at sites separated by about 100 bp is necessary for function. The binding of the first is accompanied by the uptake of some 25 extra water molecules; while the binding of the second shows the release of about 200 waters. The binding of the first appears to trigger a protein conformational change exposing extra surface area; while the binding of the second releases this extra bound water through loop formation mediated by protein interactions.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18015-06 LBM

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Histamine Release and Hydration of Granule Matrices

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: M.J. Curran Special Volunteer LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

This project was transferred to NICHD as of September 30, 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18016-03 LBM

PERIOD COVERED

October 1 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell-Cell Fusion Due to Influenza Hemagglutinin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: J. Zimmerberg Senior Research Investigator LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project was transferred to NICHD as of September 30, 1990.

ANNUAL REPORT

THE LABORATORY OF CELL BIOLOGY AND GENETICS

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory of Cell Biology and Genetics carries on a broad program of investigation into exocrine secretion and the molecular events regulating these processes. Four specific tissues have been studied: chromaffin cells, which secrete adrenaline, ATP and endogenous opiates; pancreatic beta cells, which secrete insulin; nerve terminals which secrete transmitters and colon carcinoma cells which secrete mucins and chloride. These studies were also carried out on the metabolism of ascorbic acid, with an aim to gathering insights into basic questions of nutritional needs and ascorbate functions. The following studies have been performed in pursuit of these goals.

I. Molecular Basis of Exocytosis from Chromaffin Cells and Beta Cells

- A. Pertussis toxin stimulation of catecholamine release from adrenal medullary chromaffin cells: mechanism may be by direct activation of L-type and G-type calcium channels.

We have previously shown that pertussis toxin (PTX) stimulates delayed-onset, $[Ca^{2+}]_0$ -dependent catecholamine (CA) release from bovine chromaffin cells. We now show that this effect of PTX is inhibited in part (50%) by dihydropyridine Ca^{2+} -channel antagonists nifedipine and nifedipine, and is potentiated by the dihydropyridine Ca^{2+} -channel agonist Bay K-8644. We and others have shown that pretreatment of chromaffin cells with PTX results in enhanced catecholamine secretion in response to high $[K^+]_o$, nicotine and muscarine, and here we extend these observations by showing that toxin pretreatment also enhances the secretory response to $[Ba^{2+}]_o$. All these data are consistent with the concept that PTX may act on Ca^{2+} channels. To examine the possibility of a direct action of the toxin on the voltage-gated L-type Ca^{2+} channel known to be present in these cells, we studied the effects of the toxin on whole cell Ca^{2+} currents. We found and report here that spontaneous electrical activity was considerably increased in PTX-treated cells. Our measurements of whole cell inward Ca^{2+} currents indicate that the underlying mechanism is a marked shift of the activation curve of the L-type Ca^{2+} current along the voltage axis toward more negative potentials. While treatment of the cells with PTX had no effect on L-type Ca^{2+} -channel conductance (6 nS/cell at 2.6 mM $[Ca^{2+}]_o$), PTX evoked the activation of a new class of Ca^{2+} -selective channels (5 pS in 25 mM $[Ca^{2+}]_{pipet}$), which are rather insensitive to membrane potential.

- B. Mixed Nicotinic and Muscarinic Features of Cholinergic Receptor Coupled to Secretion in Bovine Chromaffin Cells.

Acetylcholine evokes release from cultured chromaffin cells by a mechanism that is believed to be classically nicotinic. However, we found that the full muscarinic agonist oxotremorine-M (OXO-M) induced a robust catecholamine (CA) secretion. By contrast, muscarine, pilocarpine, bethanechol and McN-A-343 did not elicit any secretory response. Desensitization of the response to nicotine by OXO-M and that of OXO-M by nicotine suggest that both drugs are acting at the same receptor. Additional experiments supporting this conclusion show that nicotine-induced and OXO-M-induced secretion were similarly blocked by various nicotinic and muscarinic antagonists. In addition, secretion induced by both nicotine and OXO-M were calcium dependent, and were accompanied by robust uptake

of 45-[Ca⁺⁺]. Equilibrium binding studies showed that 3-[H] OXO-M bound to chromaffin cell membranes with a $K_d = 3.08 \times 10^{-8}M$, and a Hill coefficient of 1.0, suggesting one binding site for this ligand. Nicotine inhibited OXO-M binding in a non-competitive manner, suggesting that both ligands bind at two different sites on the same receptor. We propose that the receptor in bovine chromaffin cells that is coupled to secretion represents an unusual cholinergic receptor that has both nicotinic and muscarinic features.

C. Sigma Receptors Inhibit Nicotine Receptor-Stimulated Responses In Adrenal Chromaffin Cells

The physiological role of the putative sigma (δ) receptor is unknown. Phencyclidine (PCP) and phenothiazines with moderate to high affinities for the δ site also non-competitively inhibit [³H] perhydrohistrionicotoxin binding to the nicotinic receptor-coupled inophore (*Int. Rev. Neurobiol.*, 30:1, 1988). Since PCP also inhibits nicotine (NIC)-stimulated catecholamine (CA) release in adrenal medullary chromaffin cells (*Neuroscience* 25:687, 1988), we hypothesized that these effects were mediated by δ receptors. We now report that δ receptor ligands reversibly and non-competitively inhibit NIC-stimulated CA release from bovine adrenal chromaffin cells. These effects are both concentration-dependent and stereoselective. The inhibition of NIC-stimulated CA release is paralleled by an inhibition of NIC-stimulated increases in [Ca⁺⁺]. This inhibition by δ ligands such as (+) pentazocine (PEN) does not appear to be mediated by either opiate or dopamine receptors. Further, the inhibitory effects of (+) PEN are not observed when other secretagogues, such as histamine or barium are employed. A pharmacological profile consistent with the haloperidol-sensitive δ receptor is observed using [³H]3-(3-hydroxyphenyl)-N-(1-propyl)-piperidine (3-PPP) binding assays. Moreover, when [³H]3-PPP binding is performed in the presence of physiological salts, the apparent affinities of δ ligands were reduced to values effective in catecholamine release assays. These data demonstrate that a δ receptor modulates nicotinic receptor-stimulated responses in the adrenal chromaffin cell system. Thus, this report is the first demonstration of a cellular role for the δ receptor.

D. Osmotic Strength Differentiates Between Two Types of Calcium Transport Pathways Regulating Catecholamine Secretion From Cultured Bovine Chromaffin Cells

Calcium transport and catecholamine secretion was measured in cultured bovine chromaffin cells. Calcium ions which entered the cells following stimulation with either nicotine or 50 mM KCl (high potassium) triggered catecholamine release, but then inactivated the secretory process. The nicotine and the high potassium-induced calcium transport mechanisms were mechanistically distinct, but functionally dependent on each other. The specific evidence is that whereas the high potassium-induced Ca²⁺ influx was found to be inhibited by hyperosmotic medium, the nicotine-stimulated calcium influx was unaffected under these conditions. High potassium and nicotine-stimulated catecholamine release were also differently affected by hyperosmotic medium. While potassium-stimulated catecholamine release was profoundly inhibited by hyperosmolarity, nicotine-stimulated release was only moderately inhibited. Sequential treatments of cells with nicotine and high potassium, under isotonic physiological conditions, indicate that there is a functional, biochemical communication between the otherwise mechanistically distinct calcium channels. Calcium ions which were

found to inactivate these channels may be the basis for such communication.

E. Camp and ATP Differentially Affect Activity and Cooperativity of Lipocortin-I (Annexin-I) Driven Chromaffin Granule and PS Liposome

Lipocortin I and other members of the annexin gene family have been reported to share homology with a limited portion of the 1st nucleotide binding fold (NBF) domain of the cystic fibrosis transmembrane regulator (CFTR). We find that ATP blocks lipocortin-driven granule aggregation, but that both AMP and GTP are inactive. ATP also blocks both the rate and the extent of liposome aggregation, but leaves lipocortin cooperativity (n_H =ca. 2) unaffected. Lipocortin-I driven granule aggregation can be activated by cAMP, but not cGMP. Upon testing with PS liposomes we noted that cAMP increases the cooperativity of lipocortin-I by 2-3 fold. We thus conclude that ATP blocks activity, but that cAMP modulates protein cooperativity. These data thus appear to be consistent with the previously detected CFTR/annexin homology in the NBF domain, and suggest a parallel functional relationship as well.

F. Synexin: A Target Protein For Toxic Effects of Cyclosporin

Immunosuppressant drugs such as cyclosporin (CsA) and FK506 act on T cells and exert immune and non-immune toxic side effects by mechanisms which remain poorly understood. Both drugs are presumed to act at the nuclear level. However, recent studies on exocytosis from T cells, mast cells and beta cells from islets of Langerhans have also pointed to the actions of both drugs on exocytotic secretion at some site after calcium entry. Synexin (Annexin VII) is a calcium binding protein with calcium channel and membrane fusion properties, which has been proposed as a mediator of membrane fusion during exocytosis (Pollard, Burns, and Rojas, J., *Membrane Biology*, 117:101-112, 1990). Since synexin is thought to promote membrane contact and fusion after the entry of calcium, we considered the possibility that these drugs might be acting on synexin. We found that in the presence of calcium, both CsA and FK506 are potent inhibitors of synexin driven chromaffin granule aggregation, with K_i app values of 3.2 μ M (ca. 4 μ g/ml) and 7 μ M (ca. 5 μ g/ml), respectively. The mechanism of synexin action on the aggregation process is known to involve calcium-dependent self-association of synexin monomers into active dimers and higher order multimers, and selective binding of the active synexin molecules to target acidic phospholipids. However, 10 μ g/ml (8.3 μ M) CsA had no effect on self-association of synexin, as estimated by a quantitative light scattering assay. Neither did the drug affect synexin driven aggregation and fusion of liposomes prepared from pure phosphatidylserine. We therefore concluded that the mechanism of CsA action on synexin involved a specific aspect of the interaction of synexin with the secretory granule, per se. To help distinguish between possible principal action of CsA on either synexin or granules, we treated isolated chromaffin granules with 10 μ g/ml CsA, washed away the drug, and then tested the treated granules for competence as a synexin substrate. We found that these granules were as active as control granules, and therefore concluded that the site of action of CsA on the system was most likely on synexin. As a control, we also investigated the possible action of CsA and FK 506 on the homologous annexin, lipocortin I (annexin I). Lipocortin I also aggregates chromaffin granules in the presence of calcium, but CsA had no apparent effect. We take these data to indicate that the toxic side effects of both CsA and FK506 may be effected by action on synexin driven processes. Consistently, the range of inhibition of

synexin by CsA is at the high end of the therapeutic window for this drug. By contrast, FK506 acts as an immunosuppressant drug at 10-100-fold lower concentrations, and so is relatively free of the consequences of toxic side reactions mediated by inhibition of synexin. This insight may also prove valuable for the design of other immunosuppressant drugs in the CsA/FK506 class, since the inhibitory action of these drugs on synexin may be a useful *in vitro* index of toxicity.

G. Synexin (Annexin VII), Calcium, and the "Hydrophobic Bridge Hypothesis" for Membrane Fusion During Exocytosis

Exocytosis is the general process by which secretory granules fuse to plasma membranes and release stored enzymes, hormones or neurotransmitters to the extracellular medium. In many cell types, including chromaffin cells to which we have devoted particular attention, the process is activated by increased intracellular calcium concentration, and the resulting membrane fusion event may be facilitated by intracellular calcium binding proteins. Our recent studies have supported the concept that synexin (annexin VII) may be a compelling candidate for such a facilitating protein; and that once activated by calcium, synexin can itself drive the membrane fusion process. Based on the ability of synexin to form calcium channels, and on the results of molecular modelling and computer simulation, we have suggested that upon activation by calcium synexin binds to and penetrates acidic phospholipid-rich target membranes. Furthermore, inasmuch as synexin can also form polymers in the presence of calcium, we have suggested that activated synexin oligomers begin the fusion process by forming hydrophobic crosslinks between target membranes. We have termed this concept "the hydrophobic bridge" hypothesis for membrane fusion. Our present understanding of the mechanism of this process indicates that the intimate interactions between the hydrophobic synexin molecules and the membranes occur as follows: calcium-activated synexin molecules first make specific contact with the head groups of the acidic phospho-lipid on the bilayers. Thereafter, the highly hydrophobic protein becomes enveloped by hydrophobic fatty acid chains from the bilayer interiors. This mechanism takes advantage of the fact that the lipid chains have essentially liquid-like mobility within the confines of the bilayer, and approach the bilayer-water interface with high probability. We have calculated that fusion by this mechanism could take as little as 4 microseconds, thus allowing substantial time for other intervening biochemical and physical events.

H. Images of Synexin in Rat Brain: Heterogeneous Distribution Within Limbic Structures As Determined By Computer-Enhanced Neuro-Immunocytochemistry

Synexin (Annexin VII) is a calcium-binding protein with membrane fusion and calcium channel properties, which occurs in brain with an additional specific, cassette-exon defined domain of 22 amino acids edited into the unique N-terminal region. The exact function is not known, but we report here that an affinity purified polyclonal antibody directed against the 22 residue peptide from human brain synexin is heterogeneously distributed in the rat brain. The specific localization includes (1) hippocampal pyramidal cells in CA1 and CA3, granule cells of the dentate gyrus, and Schaffer collaterals which are axonal projections of CA3; (2) cingulate cortex, layers 2-6; (3) somatosensory cortex, layers 2-6, including heterogeneous column-like structures; (4) Thalamus, including ventromedial lateral (VLM) and ventral posterior medial (VPM) nuclei, which are motor

projections to the motor cortex; (5) axons in the corpus callosum; (6) the lateral habenula, which is primarily dopamine cells and which shares with inferior colliculus the highest intensity of deoxyglucose metabolism in rat brain; (7) the ventral aspects of the pars reticularis of the substantia nigra, which contains GABA-ergic terminals from neurons in the caudate-putamen; and (8) axons in the corpus callosum. A second polyclonal antibody against a peptide from the C-terminal domain of synexin prepared in a different animal gave nearly identical distribution in rat brain. High power views demonstrate unambiguously that synexin is enriched in neuronal somata and axons. We conclude that there seems no evidence for molecular heterogeneity in synexin distribution in rat brain, and that the distribution seems limited to specific regions of the limbic system and axons. An implication of this study is that the synexin molecule may play a role in fundamental processes underlying emotions, learning, and memory. However, the possible involvement of synexin in cognitive dysfunction associated with the aging nervous system remains to be elucidated.

I. Leucine - Induced Insulin Secretion

Leucine is known to enhance insulin secretion from islets of Langerhans, and insulin promotes leucine uptake in peripheral tissues. The present studies were designed to elucidate the effects of leucine on glucose responsiveness and stimulus secretion coupling in mouse islets of Langerhans. The effects of 20 mM leucine on insulin secretion and membrane potential were studied over a range of glucose concentrations (0.27-7 mM). Microdissected, perfused pancreatic islets from normal adult mice were used for both studies of insulin secretion and electrophysiology in order to make a close comparison between these measurements. Leucine enhanced the insulin secretion in the presence of 5.6, 11.1, and 22.2 mM glucose. In the presence of leucine, 27 mM glucose inhibited insulin secretion. In the absence of glucose-leucine did not induce electrical activity of the beta cell membrane, whereas the presence of 5.6, 11.1, and 22.2 mM glucose leucine increased spike frequency. Thus, leucine shifts both the glucose-dependent insulin secretion and electrical activity toward lower glucose concentrations. It is concluded that leucine and glucose share a common metabolic pathway (citric acid cycle) for stimulatory effects. Leucine is deaminated to form 2-ketoisocaproic acid (KIC) and produce NH_4^+ . We propose that in the absence of glucose this increases cytosolic pH, which in turn increases K^+ permeability, and inhibits electrical activity and insulin secretion.

II. Neurotransmitter Release Mechanisms

A. Endoplasmic Reticulum as a Source of Ca^{2+} in Neurotransmitter Secretion

Depolarization of the synaptosomal membrane by a rapid elevation of $[\text{K}^+]_0$ induces secretion of adenosine-5'-triphosphate (ATP) as well as the specific neurotransmitters. In addition to the classical $[\text{Ca}^{2+}]_0$ -dependent mode, we have found that ATP secretion also occurred in the absence of extracellular calcium ($[\text{Ca}^{2+}]_0$ less than 1 μM). The extent of both modalities of secretion depended on membrane potential, and the $[\text{Ca}^{2+}]_0$ -dependent secretion proceeded at a rate that was substantially smaller than that of the $[\text{Ca}^{2+}]_0$ -dependent mode at all membrane potentials examined. We propose that intracellular stores may provide the Ca^{2+} required for exocytosis in the $[\text{Ca}^{2+}]_0$ -independent mode of ATP secretion. To test this hypothesis, we searched for the presence of Ca^{2+} -release channels gated by intracellular messengers in our synaptosomal preparation.

We fused membrane vesicles from lysed synaptosomes with acidic phospholipid bilayers formed at the tip of a patch pipette and found that these membranes contained a Ca^{2+} -selective channel. The properties of this channel resemble those of the Ca^{2+} -release channel reconstituted from sarcoplasmic reticulum membrane vesicles. These include size of the single open-channel conductance (75 pS Ca^{2+} as the main current carrier), activation by adenine nucleotides (ATP), ryanodine and caffeine, and inhibition of ruthenium red.

B. Quantitative Analysis of Depolarization-Induced ATP Release from Mouse Synaptosomes: External Calcium Dependent and Independent Processes

We and others have shown previously that ATP is secreted from mouse brain synaptosomes following depolarization of the membrane by high $[\text{K}^+]_o$ and the time course can be monitored accurately by measuring the light emitted from luciferin-luciferase included in the reaction medium. In the present work we have evaluated the relative importance of $[\text{Ca}^{2+}]_o$ and membrane potential on the ATP secretion process by modelling the time course of ATP release under different conditions. After correction of the records for destruction of released ATP by synaptosomal ecto-ATPase activity, we found that ATP secretion occurs by an apparent first order process. We also established that, in addition to the classical $[\text{Ca}^{2+}]_o$ -dependent mode, ATP secretion also occurred in the absence of extracellular calcium ($[\text{Ca}^{2+}]_o < 1 \mu\text{M}$). Upon lowering the extracellular Ca^{2+} -concentration, both the rate and the extent of ATP secretion decreased. To assess the contribution of membrane potential to the release rate we measured ATP secretion at membrane potentials determined by extracellular $[\text{K}^+]_o$ (or $[\text{Rb}^+]_o$) as defined by the distribution of the carbocyanine dye, diSC₃(5). Rate constants computed from measured secretion curves revealed that this parameter was essentially independent of membrane potential in the absence of $[\text{Ca}^{2+}]_o$. Noise analysis of the light signal showed that the variance increased upon stimulation by high $[\text{K}^+]_o$, suggesting that both modes of secretion are quantal. Thus, we conclude that the rate of ATP secretion from nerve terminals depends upon Ca^{2+} entry but not on membrane potential, *per se*.

C. Integration of Cytoplasmic Calcium and Membrane Potential Oscillations

Pituitary gonadotrophs exhibit spontaneous low-amplitude fluctuations in cytoplasmic calcium concentration ($[\text{Ca}^{2+}]_i$) due to intermittent firing of nifedipine-sensitive action potentials. The hypothalamic neuropeptide, gonadotropin-releasing hormone, terminates such spontaneous $[\text{Ca}^{2+}]_i$ transients and plasma-membrane electrical activity and initiates high-amplitude $[\text{Ca}^{2+}]_i$ oscillations and concomitant oscillations in membrane potential (V_m). The onset of agonist-induced $[\text{Ca}^{2+}]_i$ oscillations is not dependent on V_m or extracellular Ca^{2+} but is associated with plasma-membrane hyperpolarization interrupted by regular waves of depolarization with firing of action potentials at the peak of each wave. The V_m and Ca^{2+} oscillations are interdependent during continued gonadotropin-releasing hormone action (>3-5 min), when sustained Ca^{2+} entry is necessary for the maintenance of $[\text{Ca}^{2+}]_i$ spiking. The initial and sustained agonist-induced Ca^{2+} transients and V_m oscillations are abolished by blockade of endoplasmic reticulum Ca^{2+} -ATPase, consistent with the role of Ca^{2+} re-uptake by internal stores in the oscillatory response during both phases. Such a pattern of synchronization of electrical activity and Ca^{2+} spiking in cells regulated by Ca^{2+} -mobilizing receptors shows

that the operation of the cytoplasmic oscillator can be integrated with a plasma-membrane oscillator to provide a long-lasting signal during sustained agonist stimulation.

D. Apamin-sensitive Potassium Channels Mediate Agonist-Induced Oscillations of Membrane Potential in Pituitary Gonadotrophs

In cultured rat pituitary gonadotrophs, gonadotropin-releasing hormone (GnRH) induces rapid hyperpolarization of the cell membrane and causes cessation of the spontaneous electrical activity present in non-stimulated cells. This initial response to GnRH is followed by slow oscillations of membrane potential (V_m) which often exhibit brief bursts of action potentials (AP) fired from the peak of the oscillations. The hyperpolarization waves are synchronous with GnRH-induced elevations of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), such that V_m maxima alternate with the peak values of $[Ca^{2+}]_i$. The V_m oscillations result from repetitive activation of apamin-sensitive K^+ channels by cytoplasmic Ca^{2+} . Thus, GnRH activation of Ca^{2+} mobilization can generate a bursting pattern of membrane potential through the activation of K^+ channels against a background of spontaneous electrical activity.

III. Protection Against Neuronal Injury

A. A Neurochemical and Behavioral Disorder Resembling Parkinson's Disease (PD) Induced in the Goldfish by MPTP Protection by 1-Deprenyl (DEPR) But Not Clorgyline (CLOR)

Parkinson's Disease (PD) has been modelled in humans and higher vertebrates by administration of the neurotoxin MPTP. We now report that PD syndrome can be uniquely elicited in the goldfish by MPTP. Mobility was measured daily, for five days, in the goldfish using the Columbus Activity Meter. The total distance traveled, time of rest, and apparent rate of motion for the fish were measured over an observation period of 5 min following drug treatments. Monoamine oxidase (MAO) activity was measured in the goldfish brain by an established radiometric procedure. As in the primate model, inhibition of MAO-B with 1-DEPR blocks the formation of the neurotoxic metabolite MPP⁺, thereby preventing motion deficits and loss of catecholamines. CLOR, a MAO-A-selective drug, failed to achieve similar results. We conclude that the MPTP-treated goldfish provides a valuable biological system for the analysis of neurotoxic and neuroprotective agents having relevance to MPTP-induced PD syndrome.

B. Extracellular Matrix Permits the Expression of Von Willerbrand's Factor, Uptake of Di-Acetylated Low Density Lipoprotein And Secretion of Prostacyclin in Cultures of Endothelial Cells From Rat Brain Microvessels

Microvascular endothelial cells from the adult brain were cultured on Matrigel and found to express many differentiated properties including secretion of prostacyclin (PGI₂) and von Willebrand's factor (vWF). Brain microvascular endothelial cells (BMECs) were purified by dextran and percoll gradients after enzymatic treatment and cultured under various conditions. BMECs that were plated on Matrigel stained positively for factor VIII-related antigen and incorporated Di-I-acetylated low density lipoprotein, whereas BMEC plated on fibronectin, gelatin, or uncoated dishes did not express any of the above properties which are characteristic of endothelial cells. vWF was measured by a sensitive ELISA in the culture media of BMECs plated on different types of matrices. Specificity of the

anti-human vWF antibodies for the rat vWF was verified by immunoabsorption on a solid phase, sodium dodecyl sulfate, and Western blot analysis. BMECs also secreted vWF into the culture media only when the cells were plated on Matrigel, and this secretion was augmented after a 6 h incubation with an interleukin-1 tumor necrosis factor- mixture, but not by lipopolysaccharide. From different matrices tested, only Matrigel permitted the secretion of PGI₂ by BMECs. Cells also proved to be sensitive to mechanical stimulation and became refractory to secretagogue if the mechanical stimulation was serially repeated. Under the best conditions, stimulation of the cells with bradykinin (1 μ M) substantially increased PGI₂ secretion. These data indicate that growth of BMECs on Matrigel in vitro permits the expression of classical endothelial cell markers in a manner similar to the behavior of these cells in situ.

IV. Studied on Approaches to Treat Cystic Fibrosis

A. A, Adenosine-receptor Antagonists Activate Chloride Efflux from Cystic Fibrosis Cells

A, adenosine-receptor-antagonist drugs such as 8-cyclopentyl-1,3-dipropylxanthine (CPX) and xanthine amine congener (XAC) are found to activate the efflux of ³⁶Cl⁻ from CFPAC cells. These cells are a pancreatic adenocarcinoma cell line derived from a cystic fibrosis (CF) patients homozygous from the common mutation, deletion of Phe-508. The active concentrations for these compounds are in the low nanomolar range, consistent with action on A₁ adenosine receptors. In addition, drug action can be blocked by exogenous agonists such as 2-chloroadenosine and also can be antagonized by removal of endogenous agonists by treatment with adenosine deaminase. Cells lacking the CF genotype and phenotype, such a HT-29 and T84 colon carcinoma cell lines, appear to be resistant to activation of chloride efflux by either drug. CFPAC cells transfected with the CF transmembrane regulator gene, CFTR, are also resistant to activation by CPX. We conclude that since, these antagonists are of relatively low toxicity and appear to act somewhat selectively, they might be considered promising therapeutic candidates for CF.

B. Flat Embedding and Immunolabelling of SW1116 Colon Carcinoma Cells in LR White: An Improved Technique in Light and Electron Microscopy

Human SW1116 colon carcinoma cells were grown on matrix-covered coverslips and flat embedded in specially prepared gelatin capsules in the hydrophilic resin LR White. Dehydration and polymerization were carried out so as to maximize preservation of antigenicity. Sections were cut perpendicular to the substratum. To visualize mucin, semithin section of SW1116 cells were stained with periodic acid Schiff (PAS) reagent for light microscopy and ultrathin sections were labelled with a monoclonal mucin antibody (Mab 19-9 and immunogold for electron microscopy. Immunofluorescence was carried out on whole cultured cells using Mab 19-9. The morphological preservation of SW1116 cells embedded in LR White was comparable to that of Epon-embedded cells. Mucin was localized on the microvillar surface of the apical plasma membrane and occasionally in intercellular spaces between adjacent cells. Mucin was also present in vesicles in the apical and lateral part, and to a lesser extent in the basal part of the cells. We conclude that this new technology significantly improves the morphological preservation of cells and tissues in LR White, while also serving to sustain the antigenicity of cellular antigens, including mucins.

C. Intrinsic Anion Channel Activity of the Recombinant First Nucleotide Binding Fold Domain of the Cystic Fibrosis Transmembrane Regulator Protein

The first nucleotide binding fold (NBF-1) from the cystic fibrosis transmembrane regulator (CFTR) has been expressed in the bacteria and found to bind ATP and to express anion channel activity when reconstituted onto a planar lipid bilayer. This evidence suggests that the NBF forms the anion-selective portion of the CFTR channel. We also found that the recombinant NBF-1 anion channel is blocked by ATP (1 mM), under which condition it appears to have a minimal conductance of ≈ 9 pS and an ohmic current-voltage relationship. We further found that the recombinant NBF-1 bearing the $\Delta F508$ mutation has nearly identical anion channel activity to that of the wild-type protein but can be distinguished from wild-type under bianionic conditions with chloride and gluconate. We conclude from these data that the anion channel activity of the recombinant NBF-1 could represent all or part of the anion conductance mechanism of CFTR and that the role of the ATP binding by the NBF could be to modulate this anion channel activity.

D. Control of Mucus Secretion By Phospholipase A Inhibitors

Cystic Fibrosis (CF) is characterized by enhanced secretion and subsequent accumulation of mucus in the airways, which is a principal cause of morbidity and mortality in the disease. CF mucus has also been found to contain high levels of eicosanoids (prostaglandins and leukotrienes, which further induce mucus secretion and thus exacerbate the pathological state. Thus CF is a disease of aberrant regulation of secretion. Phospholipase A (PLA₂) is an enzyme present in cell membranes which plays a key role in the regulation of cellular secretion and in the production of eicosanoids. It is therefore possible that this enzyme is involved in the regulation of mucus secretion, and that inhibition of this enzyme might provide a useful therapeutic avenue for CF. To test this hypothesis we have measured mucin secretion from CF-derived tracheal epithelial cells (IB3), and have studied the possible effect of 3 PLA₂ inhibitors designed and synthesized in the laboratory of S. Yedgar. Mucin secretion was measured by determination of its accumulation in the culture medium by an ELISA assay using the 19-9 antibody against the Lewis a antigen. We found that IB3 cells constitutively secrete considerable amounts of mucin (up to 1 μ g/mg cell protein/hr), and that this secretion is further stimulated by arachidonic acid. As anticipated, both basal and stimulated mucin secretion were inhibited profoundly by the PLA₂ inhibitors. We have interpreted these data to indicate that PLA₂ is involved in regulation of mucins from IB3 cells, and that these inhibitors may prove useful for control of CF-related mucus secretion.

E. Cyclic AMP-independent Secretion of Mucin by SW1116 Human Colon Carcinoma Cells Differential Control by Ca²⁺ ionophore A23187 and m Arachidonic Acid

The regulation of mucin secretion by SW1116 human colon carcinoma cells has been studied using monoclonal antibody 19-9, which has previously been used to detect mucin in the serum of cancer and cystic fibrosis patients. We found that SW1116 cells constitutively secrete considerable amounts of mucin as the predominant glycoprotein. The secretion of mucin by these cells is independent of cyclic AMP levels, but can be further stimulated by the Ca²⁺ ionophore A23187. However, arachidonic acid and its metabolites

inhibit mucin secretion. Electron microscope studies reveal that the mucin is located near the plasma membrane as well as in vesicular and lysosome-like structures. However, the secretion pathway of mucin is different than that of the lysosomal contents, since arachidonic acid, while inhibiting mucin secretion, actually activates the secretion of the lysosomal enzyme β -glucuronidase. We suggest that the mechanism of mucin secretion by SW1116 cells occurs by a pathway different from common exocytosis, and possibly by more than one pathway. The response of mucin secretion by SW1116 cells to common secretagogues resembles that of epithelial cells obtained from cystic fibrosis patients. Thus SW1116 cells are an especially interesting system for studying processes related to pathological states associated with excessive constitutive secretion of mucin, such as cystic fibrosis.

F. Multiple Potassium and Chloride Channels in the Human Colon Carcinoma Cell Line SW1116

SW1116 cells have a profound capacity for secreting mucin molecules bearing the Lewis^x epitope. Mucin molecules with the same epitope have been found to be elevated in the serum of patients with cystic fibrosis, a disease with defective ion channels. We therefore decided to study ion channels in this cell line. In the present work, we report the presence of two K⁺-channels and two Cl⁻ channels in the apical membrane of SW1116 cells. One of the K⁺-channels has a large conductance (≈ 278 pS), anomalous rectifying properties, and is inactivated rapidly. The second type exhibited a linear I/V curve (19 pS), was voltage insensitive and inactivation was not observed. In cell-attached patches, spontaneous openings of chloride channels were seen with higher frequency than previously reported in other colon carcinoma cell lines or airway epithelial cells. Inside-out experiments allowed identification of two different Cl⁻-channels (Cl⁻-1 and Cl⁻-2). Both exhibited rectification, but in opposite directions, and both were insensitive to NIPAB.

V. Ascorbate Function in Health and Disease

The goal of this work is to determine optimal ascorbic acid (vitamin C) requirements in humans. Vitamin C requirements currently are based on prevention of the clinical deficiency disease scurvy. We developed a new approach to vitamin requirements, called *in situ* kinetics, so that vitamin requirements can be based on biochemical function *in situ*. The objectives are to discover and characterize different vitamin C biochemical functions *in situ*, to learn how these functions are regulated by vitamin C concentrations, and to determine whether the relevant vitamin C concentrations can be achieved in humans.

Several model systems are used for different aspects of *in situ* kinetics. Regulation of norepinephrine biosynthesis in adrenal medullary chromaffin granules by ascorbic acid was used to test the basic concepts of *in situ* kinetics. Neutrophils were used as one model system for human tissue. Human neutrophils contain mM ascorbic acid, but its function is unknown. Before function can be understood, regulation of transport and intracellular ascorbic acid content is being studied. Human fibroblasts are another model system. Ascorbic acid is accumulated by fibroblasts for hydroxylation of proline and lysine in pro-collagen. Hydroxylation is essential for stability of collagen. Investigation of ascorbic acid transport in fibroblasts is underway. A new analytical technique for hydroxyproline is under development. The laboratory previously discovered that human lymphocytes contain mM

concentrations of ascorbic acid; its function is unknown. To solve this problem, we began experiments using the molecular genetics techniques of differential hybridization and subtraction cloning using normal human lymphocytes and human lymphocytic tumor cell lines. Another aspect of in situ kinetics is to learn what concentrations of ascorbic acid are achieved in normal humans, so that the specific enzymatic and chemical reactions which utilize the vitamin can occur. As a prerequisite, we investigated the forms of ascorbic acid in human plasma and serum, and whether ascorbic acid was protein-bound or free in the circulation. Based on the results of these studies, a long-term clinical study was begun. Its aim is to learn for the first time what concentrations of ascorbic acid are achieved in plasma and different tissues as a function of ascorbic acid.

Publications:

1. Arispe N, Rojas E, Hartman J, Sorscher E, Pollard HB. Intrinsic anion channel activity of the wild type and the d F 508 forms of the recombinant first nucleotide binding fold domain of the cystic fibrosis transmembrane regulator protein. Proceedings of the National Academy of Sciences USA, 1992;89:1539-43.
2. Block G, Henson DE, Levine M. Ascorbic acid: a new look. *Annals of Internal Medicine* 1991;114:909-10.
3. Block G, Henson DE, Levine M. Vitamin C: Biologic functions and relation to cancer. *Nutrition and Cancer* 1991;15:249-80.
4. Block G, Henson DE, Levine M. Ascorbic acid: biologic functions and relationship to cancer. *Journal of the National Cancer Institute* 1991;83:547-50.
5. Brouwer AE, Carroll PB, Atwater IJ. Effects of leucine on insulin secretion and beta cell membrane potential in mouse islets of Langerhans. University of Leiden, Faculty of Medicine, The Netherlands. *Pancreas* 1991;6(2):221-28.
6. Butler EJ, Bergsten P, Welch R, Levine M. Ascorbic acid accumulation in normal skin fibroblasts. *American Journal of Clinical Nutrition* 1991;54:1144S-46S.
7. Burns AL, Magendzo K, Srivastava M, Rojas E, Cultraro C, de la Fuente M, Heldman J, Parra C, Pollard HB. Properties and modification of recombinant human synexin (Annexin VII). *Annals of New York Academy of Sciences* 1991;635:450-51.
8. Carroll PB, Goncalves AA, Boschero AC, Tzakis AG, Starzl TE, Atwater I. Effect of the immunosuppressant FK 506 on insulin release from adult rat islets of Langerhans. *Transplantation Proceedings* 1991;23:337-39.
9. Carroll PB, Boschero AC, Li MY, Tzakis AG, Starzl TE, Atwater I. Effect of the immuno-suppressant FK506 on glucose-induced insulin secretion from adult rat islets of Langerhans. *Transplantation* 1991;51:275-78.
10. Carroll PB, Caohuy H, Lee G, de la Fuente M, Pollard HB, Atwater I. Synexin: a target protein for the toxic effects of cyclosporin A and FK 506 in endocrine cells. *Transplantation Proceedings* 1991;23:3166-68.

11. Cena V, Brocklehurst KW, Pollard HB, Rojas E. Pertussi toxin stimulation of catecholamine release from adrenal medullary chromaffin cells: mechanism maybe by direct activation of L-type and G-type calcium channels. *Journal of Membrane Biology* 1991;122:23-31.
12. Dhariwal K, Shirvan M, Levine M. Ascorbic acid regeneration within chromaffin secretory vesicles: in situ kinetics. *Journal of Biological Chemistry* 1991;266:5384-87.
13. Dhariwal K, Hartzell W, Levine M. Measurement of ascorbic acid and dehydroascorbic acid in human plasma and serum. *American Journal of Clinical Nutrition* 1991;54:712-16.
14. Dhariwal KR, Black CDV, Levine M. Semidehydro-ascorbic acid as an intermediate in norepinephrine biosynthesis in chromaffin granules. *Journal of Biological Chemistry* 1991;266:12908-14.
15. Doron DA, Jacobowitz DM, Heldman E, Feuerstein G, Pollard HB, Hallenbeck JM. Extra cellular matrix permits expression of von Willebrand's Factor, Di-I-Acetylated low density lipoprotein and prostacyclin secretion in cultures of endothelial cells from rat brain microvessels. *In Vitro* 1991;27A:689-97.
16. Eidelman O, Guay-Broder C, van Galen PJM, Jacobson KA, Turner RJ, Cabantchik ZI, Pollard HB. A-adenosine antagonists activate chloride efflux from cystic fibrosis cells. *Proceedings of the National Academy of Sciences USA* 1992;89:5562-66.
17. Etcheberrigaray R, Feidler JL, Pollard HB, Rojas E. Endoplasmic reticulum as a source of calcium in neurotransmitter secretion. *Annals of the New York Academy of Sciences* 1991;635:90-99.
18. Etcheberrigaray R, Yedgar S, Rojas E, Pollard HB. Multiple potassium and chloride channels in the human colon carcinoma cell line SW 1116. *Membrane Biochemistry* 1992;9:215-25.
19. Fiedler J, Pollard HB, Rojas E. Quantitative analysis of ATP release from mouse brain synaptosomes: calcium-dependent and calcium-independent processes. *Journal of Membrane Biology* 1992;127:21-33.
20. Goping G, Yedgar S, Pollard HB, Kuijpers GAJ. Flat embedding and immunolabelling of SW 1116 colon carcinoma cells in LR White: an improved technique in light and electron microscopy. *Microscopic Research and Techniques* 1992;21:1-9.
21. Gottesman MM, Horio M, Lelong I, Handler J, Raviv Y, Galski H, Mickisch G, Merlino G, Willingham MC, Pastan I. Function of the multidrug transporter: drug resistance as a biochemical target in cancer chemotherapy. *BY*:
22. Heldman E, Zimlichman R, Levine M, Raveh L, Pollard HB. Relationships between catecholamine secretion and distinct calcium fluxes in cultured medullary chromaffin cells. In: *Calcium Channel Modulators in Heart and Smooth Muscle: Basic Mechanisms and Pharmacological Aspects*. Abraham S, Amitai G (Editors), VCH, Weinheim/Deerfield Beach, Florida and Salaban, Rehovot/Philadelphia 1990; pp. 87-103.

23. Kuijpers AJ, Lee G, Pollard HB. Immunoelectronmicroscopy of the calcium-binding protein synexin in isolated adrenal chromaffin granules and chromaffin cells. *Annals of the New York Academy of Sciences* 1991;635:471-74.
24. Kuijpers GAJ, Lee G, Pollard HB. Immunolocalization of synexin (Annexin VII) in adrenal chromaffin granules and chromaffin cells: evidence for a dynamic role in the secretory process. *Cell and Tissue Research* 1992;
25. Kukuljan M, Goncalves AA, Atwater I. Charybdotoxin-sensitive K (Ca) channel is not involved in glucose-induced electrical activity in pancreatic beta-cells. *Journal of Membrane Biology* 1991;119(2):187-95.
26. Lee G, de la Fuente M, Pollard HB. A barium-dependent chromaffin granule aggregating protein from bovine adrenal medulla and other tissues. *Annals of the New York Academy of Sciences* 1991;635:477-79.
27. Levine M, Dhariwal KR, Washko P, Wang Y-H, Welch R, Bergsten P, Butler EJ. Ascorbic acid and in situ kinetics: a new approach to vitamin requirements. *American Journal of Clinical Nutrition* 1991;54:1157S-62S.
28. Magendzo K, Shirvan A, Pollard HB, Burns AL. Tissue regulated alternative splicing of synexin in mRNA. *Annals of the New York Academy of Sciences* 1991;635:483-84.
29. Paul IA, Rojas E, Youdim MBH, De Costa B, Skolnick P, Pollard HB, Kuijpers GAJ. Sigma receptors modulate of nicotinic receptor function in adrenal chromaffin cells. In: Multiple Sigman and PCP Receptor Ligands: Mechanisms for Neuromodulation and Neuroprotection. Kamenka J-M, Domino EF (Editors), NPP Books, Ann Arbor, Michigan 1992; 1-5.
30. Pollard HB, Rojas E, Pastor RW, Rojas EM, Guy HR, Burns AL. Synexin: molecular mechanism of calcium-dependent membrane fusion and voltage-dependent calcium channel activity. "Evidence in support of the "Hydrophobic Bridge Hypothesis" for Exocytotic Membrane Fusion." *Annals of the New York Academy of Sciences* 1991;635:328-51.
31. Pollard HB, Guy HR, Arispe N, de la Fuente M, Lee G, Rojas EM, Pollard JR, Sristava M, Zhang-Keck Z-Y, Merezhinskaya N, Caohuy H, Burns AL, Rojas E. Calcium channel and membrane fusion properties of synexin and other members of the annexin gene family. *Biophysics Journal* 1992;62:19-22.
32. Rojas E, Arispe N, Haigler HT, Burns AL, Pollard HB. Identification of annexins as calcium channels in biological membranes. *Bone and Mineral* 1992;17:214-18.
33. Stojilkovic SS, Kukuljan M, Iida T, Rojas E, Catt KJ. Integration of cytoplasmic calcium and membrane potential oscillations maintains calcium signaling in pituitary gonadotrophs. *Proceedings of the National Academy of Sciences USA* 1992;89(9):4081-85.
34. Washko P, Rotrosen D, Levine M. Accumulation of ascorbic acid in human neutrophils. *American Journal of Clinical Nutrition* 1991;54:1221S-37S.

35. Washko PW, Welch RW, Dhariwal KR, Wang Y, Levine M. Ascorbic acid and dehydroascorbic acid analysis in biological samples. Analytical Biochemistry 1992;204:1-14.
36. Yedgar S, Eidelman O, Malden E, Roberts D, Etcheberigarrrary R, Goping G, Pollard HB. Cyclic AMP independent secretion of mucin by SW1116 human colon carcinoma cells: differential control by Ca^{2+} ionophore A23187, and arachidonic acid. Biochemical Journal 1992;283:421-26.
37. Youdim MBH, Dhariwal K, Levine M, Markey CJ, Markey S, Caohuy H, Adeyemo OM, Pollard HB. MPTP-induced "Parkinsonism" in the goldfish. Neurochemistry Internals 1992;20(suppl):275S-78S.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 21008-25 LCBG

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cytogenetics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J.H. Tjio

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 21019 10

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of Hormone and Transmitter Secretion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Harvey B. Pollard, Chief, LOBG, NIDDK, Others: G. Lee, Ph.D., Res. Chemist; E. Rojas, Ph.D., VS; I. Abaster, Ph.D., IB; M. Levine, M.D., Sen. Inv., Med. Off.; A. Burns, Ph.D., IB; M. Srivastava, Ph.D., SSF; C. McCutchen, Ph.D., Res. Phys.; G. Kuipers, Ph.D., VA; M. Kukuljan, M.D., WF; G. Goping, BS Tech.; P. Carroll, M.D., SV; P. Washko, DDS, SV; D. Doron, M.D., SV; M. Adeyemi, Ph.D., SV; F. Vargas, DDS, HMA K. Tharival, Ph.D., SSF; R. Welch, Ph.D., SV; R. Yu, Ph.D., SV; Y-H Wang, M.D., WF; Z. Zhang-Keck, Ph.D., SF; H. Cachay, B.S., Biologist; C. Guy-Broder, BS, Biologist; C. Boschero, Ph.D., VS; N. Ariape, Ph.D., VA; Y. Raviv, Ph.D., VA; E. Olivares, M.D., VA; L. Vergara, M.D., VS; S. Pope, M.S. Microbiologist; O. Eidelman, Ph.D., SV; N. Mereshinskaya, Ph.D., VA.

COOPERATING UNITS (if any)

Dipak Banerjee, Ph.D., University of Puerto Rico; Harry Haigler, Ph.D., University of California at Irvine, Jack Cohen, Georgetown University, John Hallenbeck, USHS

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Laboratory of Cell Biology and Genetics

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Section on Cell Biology and Biochemistry

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TOTAL STAFF YEARS:

8.0

PROFESSIONAL:

7.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our work continues to focus on the processes leading to fusion between granule and plasma membranes during exocytotic secretion from cells, including chromaffin cells, beta cells from Islets of Langerhans, nerve terminals, and mucin secreting cells from tissues affected by cystic fibrosis. The contact and fusion processes during secretion may be mediated by the calcium binding protein synexin (annexin VII). We have learned that this protein is a target for the immunosuppressant drugs, cyclosporin and FK506, and that it is heterogeneously distributed within the nervous system and elsewhere. The annexin lipocortin I (annexin I) may be the mediator of barium activated secretion, and both synexin and lipocortin I activity appear to be modulated by nucleotides, including cAMP and ATP. Endonexin II (annexin V) has been shown to make up a substantial portion of the matrix vesicles used to create new bone, and to be the pathway for allowing calcium to be laid down at the appropriate site. Further studies on secretion of neurotransmitters have indicated that secretion can occur independently of extracellular calcium stores, and occurs at a much slower rate than conventional secretion. Pituitary gonadotrophs also secrete in response to a calcium signal, but in this case oscillations in membrane potential, run by an apamin-sensitive K⁺ channel cause oscillations in calcium concentration within the cells. MPTP, a neurotoxin which causes a Parkinsonian syndrome in man, also causes a similar syndrome in the goldfish, which like the human disease can be defended against with the MAO-B inhibitor L-deprenyl. The nucleotide binding fold portion of the CFTR has been expressed and shown to be a nucleotide-gated anion channel in planar lipid bilayers. Two A₂-adenosine antagonists have been found to activate chloride efflux from cystic fibrosis cells, and DPCPX is being developed for a clinical trial. A macromolecular phospholipase-A inhibitor has been shown to inhibit mucin secretion from cystic fibrosis and control cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 22001

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Vitamin C: Biochemistry, Molecular Biology and Human Requirements

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Mark A. Levine	Senior Investigator	LCBG:NIDDK
Other:	Kuldeep Dhariwal	Senior Staff Fellow	LCBG:NIDDK
	Philip Washko	Staff Fellow	LCBG:NIDDK
	Richard Welch	IRTA	LCBG:NIDDK
	Yaohui Wang	Visiting Fellow	LCBG:NIDDK

COOPERATING UNITS (if any)

L. Cantilena (USUHS, Bethesda), C. Cantilena (Transfusion Medicine, CC, NIH), D. Rotrosen (LHD, NIAID, NIH), J. Butler (HGB, NICHD, NIH), P. Bergsten (Univ. Uppsala, Sweden), L. Helman (PB, NCI, NIH) and M. Vasquez (PB, NCI, NIH)

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TOTAL STAFF YEARS:

4

PROFESSIONAL:

4

OTHER:

-

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Optimal amounts of ascorbic acid (vitamin C) for maintaining human health are unknown. As a unique means to address this problem we developed the concept of in situ, in which the principles of reaction kinetics are applied to vitamin C dependent reactions in situ. We studied catecholamine biosynthesis in secretory vesicles to test the principles of in situ kinetics. We found that ascorbic acid provides single electrons via transmembrane electron transfer to the intravesicular enzyme dopamine beta-monooxygenase. Ascorbic acid outside vesicles transfers electrons to reduce the free radical intermediate semidehydroascorbic acid within vesicles; the free radical is formed as ascorbic acid within vesicles transfers single electrons to the monooxygenase. Electron transfer in situ occurs at V_{max} under physiologic conditions. These experiments validated the principles of in situ kinetics. We previously discovered that ascorbic acid is accumulated by human neutrophils in mM concentration. We recently found that the physiologic structural analog glucose exquisitely regulates ascorbic acid transport. Experiments are underway to characterize the mechanism of glucose regulation. We discovered that human lymphocytes also contain mM concentrations of ascorbic acid. To learn vitamin C function, experiments using differential hybridization and subtraction cloning are underway. Ascorbic acid accumulation in human fibroblasts was characterized as a prelude to understanding how different concentrations of the vitamin regulate proline hydroxylation. Clinical goals are to learn how much vitamin C is found in humans as a function of ingestion, so that ascorbic acid dependent reactions can occur. In addressing prerequisite issues we found that ascorbic acid in normal human plasma and serum is free and not protein bound. Ascorbic acid circulates only in the reduced form. With these results an intensive clinical trial was proposed and approved to learn how ascorbic acid ingestion regulated plasma and tissue concentrations.

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

I. POLYAMINES

In our older work we have shown that putrescine, spermidine, and spermine are polyamines that are widely distributed in biological materials. We have developed methods for their detection, and elucidated the biosynthetic pathways and metabolism of these amines. We have been particularly interested in studying the physiological function of these amines, and for this purpose have constructed mutants (both point and deletion) in both *Escherichia coli* and *Saccharomyces cerevisiae*. We have also constructed numerous multicopy plasmids containing the genes for the biosynthetic pathways, and have used these plasmids for the overexpression and study of the biosynthetic enzymes.

In last year's report we described our results with a deletion mutant in the *spe2* gene of *S. cerevisiae*. We had shown that the *spe2* gene codes for the enzyme, S-adenosylmethionine decarboxylase, and, since, as we have shown previously, decarboxylated S-adenosylmethionine supplies the aminopropyl moiety in the biosynthesis of spermidine and spermine, a $\Delta spe2$ strain cannot make spermidine and spermine. After growth in amine-free media these cells show an absolute requirement for spermidine or spermine for growth and, when examined microscopically, show a striking morphology; namely, marked increase in volume, many large intracellular vesicles, reduced budding, abnormal distribution of chitin and actin. These effects were of additional interest because comparable pleiotropic effects are found in various *cdc* (cell division cycle), *actin*⁻, and *chitin*⁻ mutants of *S. cerevisiae*.

In our current work we show that spermidine and/or spermine are critically involved in the maintenance of the cell envelope and in the integrity of mitochondria. Our results show that spermidine and spermine are very important in protecting these moieties from damage resulting from various stress conditions including oxygen and superoxide radicals.

In order to test for the integrity of the cell envelope, we developed a technique using resistance to lysine by added Sarkosyl. With this technique we have shown that the cell envelope is unstable and permeable in the polyamine-deficient cells. Thus, we have found that the polyamine-deprived cells are strikingly sensitive to treatment with Sarkosyl, while polyamine-supplemented cells are unaffected by Sarkosyl under the same conditions. Incubation in 1M mannitol exerts a striking protective effect both on the inhibition of growth and on the sensitivity to Sarkosyl. In addition, the cells that are partially depleted of polyamines are dramatically sensitive to incubation at 39° or to incubation in 95% O₂-5% CO₂, resulting in lysis of the cells even in the absence of Sarkosyl. Mutants defective in superoxide dismutase are particularly sensitive to polyamine-deficiency.

Closely related to the above effects of polyamines on the stability of the cell envelope is our finding that the mitochondria from polyamine-deficient cells are defective, and cannot utilize glycolate. This loss of functional mitochondria is irreversible.

Suppressor mutations have been isolated that permit 50-100 fold increase in the growth of polyamine-deficient mutants. These mutations are being mapped and characterized.

. Drs. D. Balasundaram, C. W. Tabor, and H. Tabor

Another method of abolishing the biosynthesis of spermidine and spermine is to study strains that have a deletion in *spe1*, the gene coding for ornithine decarboxylase. (Such strains have been prepared in this laboratory in the past in collaboration with Dr. Xie.) Such strains cannot make putrescine, and hence cannot synthesize either spermidine or spermine. To our surprise we have found that *spe1* mutants are much more susceptible to polyamine deprivation than *spe1 spe2* mutants, even though both strains lack putrescine, spermidine and spermine. These results indicate a hitherto undescribed toxicity of decarboxylated S-adenosylmethionine (in the *spe1* mutant), which may be of significance in evaluating therapeutic results with inhibitors of ornithine decarboxylase.

We have also been studying a mutant of *S.cerevisiae* that is defective in the gene (*spe3*) coding for the enzyme putrescine aminopropyl transferase. This enzyme is also required for the biosynthesis of spermidine, but the mechanism of the requirement is different from the mutants in the *spe2* gene.

. Drs. D. Balasundaram, N. Hamasaki, C. W. Tabor, and H. Tabor

A very sensitive chromatographic method has been developed for the determination of hypusine (a spermidine derivative) in yeast protein, and is being used to follow the loss of this compound during the development of polyamine deficiency.

. Drs. C. W. Tabor and D. Balasundaram with Dr. D. Liu
(Division of Biochemical Biophysics, OBR, FDA)

We are continuing our studies on the regulation of ornithine decarboxylase in yeast. By complementation studies we are localizing the regulatory *spe40* mutation; this is very intriguing since it is closely linked to the *spe1* mutation, and surprisingly both suppresses the effects of the *spe1* mutation on growth and causes a complete defect in the synthesis of spermine synthase.

. Dr. C. W. Tabor

II. YEAST RNA VIROLOGY

We study the mechanism of propagation of the L-A dsRNA virus of the yeast *Saccharomyces cerevisiae*, the host genes which it uses for this purpose and the mechanisms by which the host limits viral propagation to prevent viral cytopathology. We find that the N-terminal 1/4 of the L-A *pol* gene is needed for encapsidation of viral RNA, but that only *gag* is needed to assemble viral particles. We have found that *pol* encodes three ssRNA binding regions, one of which is cryptic, its activity being inhibited by a region C-terminal to this binding region. One of the ssRNA binding regions of *pol* is within the region necessary for packaging, suggesting that this is its *in vivo* role. The others are likely to be involved in RNA replication and transcription. The assembly process requires the MAK3 N-

acetyltransferase that acetylates the N-terminus of gag. Stable particle formation also requires the MAK10 protein, particularly if the particles are completely full.

. Drs. J. C. Ribas, T. Fujimura, J. C. Tercero, Y.-J. Lee and R. B. Wickner

The six SK1 genes comprise a host system to limit the replication of three unrelated dsRNA and ssRNA replicons. We have obtained evidence that they do this by limiting the translation of uncapped messages.

. Drs. W. R. Widner and R. B. Wickner

We have isolated mutations in several chromosomal genes that result in substantial increases in the efficiency of the -1 ribosomal frameshifting that L-A uses to synthesize its gag-pol fusion protein. We find that the efficiency of frameshifting is critical to viral propagation, suggesting that drugs affecting this process may be useful against retroviruses.

. Drs. J. D. Dinman and R. B. Wickner

The [URE3] non-Mendelian genetic element of yeast requires for its propagation the URE2 chromosomal gene. We have evidence that [URE3] is a prion of yeast.

. Dr. R. B. Wickner

III. NUCLEIC ACIDS

We are studying the *E. coli* bacteriophage T4 as a model system for duplex DNA replication. Efficient DNA replication *in vitro* requires at least ten purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43 product), gene 32 helix destabilizing protein, the gene 44/62 and 45 polymerase accessory proteins, the genes 41 and 61 proteins that function together as a primase and as a DNA unwinding enzyme (helicase), gene 59 protein that stimulates the primase-helicase, RNase H, and DNA ligase.

Assembly of the polymerase and accessory proteins on the primer-template. We have previously used cross-linking of proteins to photo-activatable residues at specific nucleotides in the primer to determine the position of polymerase and each of the accessory proteins on the primer, and to show that ATP binding was sufficient to add the three accessory proteins to the primer, but that ATP hydrolysis, as well as the three accessory proteins, were required to clamp polymerase to the primer in a replication complex stable enough for processive DNA synthesis. These studies were carried out with a 34 nucleotide primer annealed to a large (>5000b) circular single-stranded DNA template. This large primer-template is not suitable for studies to determine the location of the polymerase and accessory proteins on the template strand, for gel mobility shift assays to look steps in the assembly of the polymerase holoenzyme on the DNA, or for pre-steady state kinetic analysis of synthesis by the polymerase and accessory protein complex. In order to design a small primer-template that could be used for these studies, we are determining the minimum size of the primer and template strands that will allow the proper assembly of the polymerase accessory protein complex, as judged by cross-linking of the proteins and analysis of the DNA products. We find accessory protein dependent cross-linking of polymerase to a 34b primer annealed to a 104b template, with 35

nucleotides of single-stranded DNA in front of and behind the primer, but not to the same primer annealed to a 69b template. It is not yet clear whether the kinetics of the assembly of the complex on the 34:104 mer are the same as those on the larger circular template.

We have used gel-mobility shift assays of protein-DNA complexes cross-linked with glutaraldehyde to demonstrate a complex of the gene 45 polymerase accessory protein and polymerase on a 36:64mer primer-template. However, the formation of the complex of 45 protein and polymerase on this DNA requires very high concentrations of 45 protein, and is not dependent on the 44/62 polymerase accessory protein or ATP. Moreover, we find no evidence for a complex containing polymerase whose formation requires all three accessory proteins and ATP hydrolysis. Thus this gel mobility shift technique also suggests that the 34:64mer is too small for the proper assembly of the T4 polymerase holoenzyme on the primer.

. Drs. T. L. Capson and N. G. Nossal

Construction and analysis of mutations in T4 DNA polymerase. T4 DNA polymerase has a single polypeptide of 898 amino acids that has remarkable sequence similarity in several collinear regions with a large family of DNA polymerases which includes the eukaryotic cellular polymerases α , δ , and ϵ , as well as the herpes and vaccinia viral polymerases. We are trying to determine the function of these conserved regions, and to identify regions of the polymerase important for its interaction with the other T4 replication proteins, by *in vitro* mutagenesis of the cloned T4 polymerase gene. We have developed a simple protein purification procedure that yields homogeneous polymerase in a single day, which we are using to characterize the altered enzymes.

T4 DNA polymerase has a 3'→5' exonuclease that acts as a proofreader to remove incorrect nucleotides inserted by the polymerase. In order to assess the contribution of the editing exonuclease to the fidelity of DNA replication, we have constructed a polymerase lacking this nuclease by site directed mutagenesis of a conserved amino acid in the N-terminal region. This T4 DNA polymerase has no measurable exonuclease activity and retains a polymerase activity whose rate of synthesis and interaction with the other T4 replication proteins is very similar to that of the wild-type polymerase. In collaborative studies, Michelle West Frey and Stephen Benkovic (Pennsylvania State University) are carrying out a pre-steady state kinetic analysis of this mutant polymerase. We have also constructed a T4 bacteriophage with the exonuclease defective polymerase gene, and in preliminary studies, find that its rate of mutation at the single locus tested is about 1000 fold higher than that of the wild type phage.

. Drs. N. G. Nossal and T. L. Capson, in collaboration with M. W. Frey and Dr. S. J. Benkovic (Pennsylvania State University)

Function of the T4 gene 59 primase-helicase assembly factor. The gene 59 protein stimulates DNA synthesis by increasing the rate at which the gene 41 protein is loaded on the DNA template. 41 Protein acts as a DNA helicase, and is also essential (along with the 61 protein) for the synthesis of the RNA primers that initiate new discontinuous fragments on the lagging strand. We have recently found that addition of 59 protein to the seven protein T4 replication system (polymerase, three polymerase accessory proteins, single-stranded DNA binding protein, 41 and 61 protein primase-helicase) gives a ten-fold increase in synthesis on linear duplex T4 DNA and on other large double stranded templates. In contrast to

synthesis by the seven protein system that begins at nicks in double-stranded DNA, 59 protein-dependent synthesis is not inhibited by adding T4 DNA ligase to seal nicks in the duplex. We are currently trying to determine how and where synthesis on the duplex DNA is initiated in the 59 protein-dependent reaction.

. Dr. N. G. Nossal

T4 RNaseH. We have previously identified a T4 gene encoding an RNaseH, and cloned the gene in a T7 expression vector. We have devised a rapid two step procedure that gives homogeneous RNaseH from cells containing this plasmid. Although we have shown that purified T4 RNaseH efficiently removes RNA primers *in vitro*, it is not yet clear whether it plays this role *in vivo*. T4 RNaseH is encoded between T4 genes 33 and 34, in a region where no conditionally lethal mutations have been found. Starting by *in vitro* mutagenesis of the RNaseH plasmid, we have constructed a T4 phage with an extensive deletion within the RNaseH gene. Preliminary results indicate that the burst size of the RNaseH deletion phage is somewhat reduced in a wild type *E. coli* host, and is extremely low in a host that is defective in both *E. coli* RNaseH and the 5'→3' exonuclease activity of polymerase I, the two *E. coli* enzymes that are required to remove the RNA primers in the host. Thymidine incorporation into DNA by the RNaseH deletion phage is also reduced in the mutant host. We are presently characterizing the DNA intermediates that accumulate during replication in the absence of the phage and host RNaseH enzymes.

. Drs. L. J. Hobbs and N. G. Nossal

Structure of the T4 DNA replication proteins. We now have plasmids which give high level expression of nine of the T4 replication proteins, and have developed methods to purify large quantities of each of these proteins. We have begun a collaboration with Craig Hyde, Laboratory of Structural Biology, NIAMS, to try to crystallize these proteins.

. Dr. N. G. Nossal in collaboration with Dr. C. Hyde, LSB, NIAMS

Transcriptional activation by the bacteriophage T4 protein MotA. During T4 infection, transcriptional control is accomplished by phage-encoded factors that alter the specificity of the host RNA polymerase. We are studying the activation of transcription from T4 middle promoters by the T4 factor MotA. We have established an *in vitro* transcription system composed of partially purified RNA polymerase isolated after T4 infection and MotA, isolated from a clone. Using our *in vitro* system, we are investigating the action of MotA at the T4 middle promoters, PuvxX and PsegA. We have found that transcription from PuvxX *in vitro* is sensitive to the addition of the polyanion heparin before but not after the addition of MotA. This result suggests that MotA is needed to form an open complex at PuvxX (the complex in which polymerase has partially unwound the promoter) since open complexes are known to be heparin resistant. We also find that in the presence of heparin, more MotA-dependent transcription from PuvxX is seen using glutamate as the major anion than is observed with Cl⁻. This result suggest that glutamate stabilizes the protein-PuvxX interactions, and is consistent with the fact that substitution of glutamate for Cl⁻ enhances protein-nucleic acid interactions in other reactions.

We have partially purified and characterized a mutant MotA protein, MotA21 in which the first 8 amino acids of MotA (MSKVITYII) have been changed to

MNNLVAKHNFN. Using gel retardation assays with PuvxS, we find that MotA21 binds to this promoter nearly as well as MotA. However, MotA21 does not support transcription from PuvxS *in vitro*. These results are consistent with the motA21 mutation affecting a domain of MotA that is involved in transcriptional activation rather than DNA binding.

. Drs. R. March-Amegadzie and D. M. Hinton

Further characterization of T4 *segA*, a gene whose product is similar to endonucleases encoded by mobile group I introns. We have previously identified a new T4 gene, called *segA*, which encodes a protein that shares regions of similarity with a family of group I intron-encoded endonucleases present in fungi and phage. These proteins are required for the movement ('homing') of the intron DNA into its intronless gene, cutting at or near the site of intron insertion. Like these endonucleases, *SegA* is a Mg^{++} -dependent DNA endonuclease which cuts with some sequence specificity, but it does not appear to be associated with an intron. We have used primer extension analyses to determine the exact positions of cutting by *SegA* at three preferred sites. While these three sites share some sequence elements, a consensus sequence cannot be deduced. This suggests that either *SegA* does not require a set of invariant bases or it recognizes some feature of the DNA other than sequence alone. Using polymerase chain reaction, we have screened the genomes of 30 T-even like phage. We find that all of these have large deletions in the region where T4 contains *segA*. Our sequence analysis of T2, a phage which is highly related to T4, has revealed that the *segA* gene is indeed missing in the T2 genome. The uneven distribution of the *segA* gene in the genomes of T-even phages is consistent with the idea that the *segA* gene was or is mobile.

. Drs. M. Sharma and D. M. Hinton

IV. GENOMIC STRUCTURE AND FUNCTION

L1 DNA (long interspersed repeated DNA, LINE 1 DNA) is a self replicating parasite of mammalian genomes and accounts for at least 10-20% of mammalian DNA. L1 elements contain a transcriptional regulatory sequence at the left end, two highly conserved genes (one of which encodes a reverse transcriptase), and a guanine-rich polypurine:polypyrimidine sequence near the right end. Transposition of L1 elements is a frequent cause of polymorphism in mammals including humans where it has caused genetic defects. We have been studying the L1 family of rats and describe below our recent findings.

We previously showed that the L1 polypurine:polypyrimidine sequence adopts a series of abnormal DNA structures which we have now found to affect iNA replication, recombination and transcription *in vivo*. For example, plasmids containing polypurine sequences have greatly reduced rates of DNA replication in both bacteria and mammalian cells. On the other hand, recombination is stimulated about 10-fold by these sequences. In bacteria, these plasmids are found as multimers, a byproduct of *recA*-independent recombination. These plasmids are unable to replicate in *recBC*, *sbcA* mutants possibly because the cells are unable to resolve very large multimers. Preliminary experiments in human cells indicates that non-B DNA structures increase plasmid recombination frequencies about 10-fold over background levels. And finally, the L1 polypurine sequences dramatically reduce the activity of the SV40 promoter *in vivo*. Work is now underway to

study the effect of these sequences on expression of a bacterial gene that is under the control of a supercoil dependent promoter (gyrA).

While doing this work we have developed a very simple and quick method for preparing plasmid DNA from single colonies suitable for DNA sequencing.

. Drs. R. Howell and K. Usdin

Structural and transcriptional analysis. We previously showed that the left-most 610 bp of rat L1 DNA resembles a transcriptional regulatory region and can stimulate the activity of a gene fused to it. No stimulation occurs if the L1 regulatory sequence is oriented in the opposite direction that it is in the L1 element. We now have found that this stimulation is due to the activation of a cryptic promoter located 5' to the L1 sequence and that the L1 sequence also activates known promoters situated 5', but not 3', to it. This rather novel transcriptional process ensures the production of a full length L1 transcript which would be necessary for replication by the L1 encoded reverse transcriptase. The L1 promoter is unusual in that it does not contain binding sites for any of the known general transcription factors. Nonetheless, DNA binding experiments show that three different regions of the L1 regulatory region can form specific complexes with partially purified nuclear factors present in either rat or primate cell lines. Deletion of the protein binding regions indicates that one of them binds a novel transcriptional stimulatory factor which we are now characterizing.

. Drs. B. Hayward, M. Zavenelli, and A. V. Furano

We have continued our previous work that demonstrated: (1) that during evolution L1 elements have undergone periodic amplification followed by periods of quiescence, and (2) that these amplified products persist in the genome. Therefore, much more of the genome than was hitherto appreciated is comprised of the products of previous amplification events, and secondly, the mammalian genome has been repeatedly assaulted and altered by the insertion of thousands of L1 elements. Our discovery of ancient amplification events also provided a novel and powerful tool for determining the evolutionary relationships between modern rodent species and, in collaboration with Dr. François Catzefflis, (Institute of Evolutionary Science in Montpellier, France) we have resolved several vexing problems in rodent phylogeny. In addition, these studies showed that amplification of L1 elements occurred during speciation. In similar studies on modern rat L1 elements we have identified a probably still active clade of L1 elements. We are now trying to isolate an intact and functional member of this clade. In the course of this work we have sequenced regions of the D-loop region of the mitochondrial DNA of a number of rodents. Data from these experiments are proving useful in resolving a number of other taxonomic problems involving the genus *Rattus*.

. Drs. A. V. Furano, B. Hayward, and K. Usdin

V. MEMBRANE STUDIES OF *ESCHERICHIA COLI*

Aldoheptose Biosynthesis. *E. coli* K-12 aldoheptose (i.e., L-glycero-D-mannoheptose) mutants carrying the *cysE-pyrE* linked mutation *rfaD* or *rfa-2*, were previously isolated and genetically characterized. These mutations result in L-glycero-D-mannoheptose deficient Lipopolysaccharide (LPS). Thus, the *rfaD* and *rfa-2* mutants produce a truncated LPS that is primary a

lipid A-2-keto-3-deoxyoctulosonic acid structure (i.e., chemotype Re). These mutants possess an outer membrane (OM) that is characteristically more permeable to a number of hydrophobic agents. The molecular genetics and biology of the *rfaD* gene has been completed and reported by this laboratory. An efficient *rfaD* gene expression system and a two-step purification protocol for the *rfaD* gene product (ADP-L-glycero-D-mannoheptose-6-epimerase) was developed. The enzyme thus purified was found to be greater than 95% pure, and it was highly active. Key kinetics and physical characterization studies of the epimerase are complete. Preliminary crystals of the epimerase have been obtained. Structural and functional homology of the *Pseudomonas aeruginosa rfaD* gene and its *E. coli* counterpart is indicated. The second mutation, designated *rfa-2*, has been exploited to define the *rfa-2* gene, resolve its sequence and flanking nucleotides, as well as determine its physical location on the *E. coli* chromosome relative to the *rfaD* gene. A *rfa-2-6xHISTAG* gene fusion has been constructed to facilitate the purification of the *rfa-2* gene product. Interspecific complementation studies demonstrated that the *E. coli* K-12 *rfa-2* gene complements the chemotype Re LPS mutant of *S. typhimurium*, designated *rfaC*. The role of the *rfa-2* gene product as a heptosyl transferase is under investigation.

. . . . Drs. W. G. Coleman, Jr., L. Chen and L. Ding

VI. ENZYME STRUCTURE AND FUNCTION SECTION

Tryptophan synthase is a multienzyme complex that catalyzes the final two reactions in the biosynthesis of L-tryptophan. We are using tryptophan synthase as a model system to understand how protein-protein interaction affects catalysis, metabolite channeling, and ligand-dependent site-site interactions.

Structure and Function of Wild Type and Mutant Forms of the Tryptophan Synthase $\alpha_2\beta_2$ Complex from Salmonella typhimurium. The crystallographic structure of the wild type tryptophan synthase $\alpha_2\beta_2$ complex is now refined to 2.2 Å resolution. Lysine-87 forms a Schiff base with pyridoxal phosphate in the active site of the wild type β subunit. Changing lysine-87 to threonine produces an inactive mutant $\alpha_2\beta_2$ complex that forms enzyme-substrate intermediates very slowly. Our spectroscopic and kinetic results provide evidence that lysine-87 serves critical roles in transamination, catalysis, and product release. The mutant $\alpha_2\beta_2$ complex forms very stable enzyme-substrate intermediates that have been analyzed by x-ray crystallography. Two new crystallographic structures of this mutant form of the $\alpha_2\beta_2$ complex containing bound L-serine or L-tryptophan reveal important information about the substrate binding site of the β subunit.

. . . . Drs. E. W. Miles and Z. Lu with Drs. C. C. Hyde, K. Parrish and D. R. Davies (LMB, NIDDK).

Subunit Communication in the Tryptophan Synthase $\alpha_2\beta_2$ Complex. An important problem in the elucidation of the allosteric mechanism is the structural basis for ligand-mediated communication between topologically distinct binding sites. We have probed the allosteric properties of the tryptophan synthase $\alpha_2\beta_2$ complex by studies using site-directed mutagenesis and limited proteolysis. Our results provide evidence that a flexible loop in the α subunit is important for ligand binding and for communicating the effects of ligand binding from the α subunit to the β subunit in the $\alpha_2\beta_2$ complex. A residue in the α subunit loop (threonine-183) plays a critical

role in modulating the enzymatic activity of the β subunit in the $\alpha_2\beta_2$ complex. Two α subunit residues that are located in the interaction site between the α and β subunits (proline-57 and proline-132) play critical roles in mutual subunit interaction and activation. Mutation of glutamate-49, aspartate-60, or glycine-51 in the α subunit inhibits the transition of the α subunit from open to a closed form and alters the kinetics of metabolite channeling.

. Drs. E. W. Miles, X. Yang, and S. B. Ruvinov with Dr. K. Yutani (Protein Institute, Osaka University, Japan) and Drs. P. Brzovic and M. F. Dunn (University of California, Riverside).

Conformational States of the β Subunit. Evidence that the β subunit can exist in two or more conformational states is provided by our studies of the reaction specificity and of substrate-induced inactivation of several forms of the β subunit. We propose that the wild type β subunit undergoes a conformational change upon association with the α subunit that alters the reaction specificity. Furthermore, several active site and tunnel mutants of the β subunit do not undergo the same conformational change upon subunit association. Our finding that the dimeric wild type β subunit is only 50% inactivated by β -chloro-L-alanine can be explained by the presence of two conformers of the β subunit in solution: one conformer is rapidly inactivated by β -chloro-L-alanine and the other conformer is not inactivated. This putative mechanism is supported by investigations using steady-state kinetics and spectroscopic and electrophoretic methods.

. Drs. S. A. Ahmed, E. W. Miles, and S. B. Ruvinov with Drs. P. Brzovic and M. F. Dunn (University of California, Riverside).

Thermal Stability of Tryptophan Synthase. We are investigating the effects of subunit association and of ligands on thermal unfolding and on thermal inactivation of tryptophan synthase. Differential scanning calorimetry studies of the thermally-induced unfolding of the $\alpha_2\beta_2$ complex show two well resolved endotherms at 51°C and at 80°C. These endotherms correspond to thermal unfolding of the α and β domains, respectively. Light scattering measurements confirm that α chains unfold without disruption of α - β contacts. Thermal inactivation studies reveal that ligands that promote subunit association (L-serine, L-tryptophan, or D-tryptophan alone or in combination with α -glycerol 3-phosphate) raise the inactivation temperature of the α subunit in the $\alpha_2\beta_2$ complex.

. Drs. E. W. Miles and S. B. Ruvinov with Drs. A. Ginsburg and D. P. Rameta (LB, NHLBI)

VII. ENDOCRINE BIOCHEMISTRY

The complex role of the cytoskeleton in the intracellular traffic of cholesterol from droplet to mitochondrion involves not only microfilaments and microtubules (as we have previously shown), but also involves the participation of intermediate filaments, as we now show for the first time. The disruption of intermediate filaments with acrylamide enhances steroidogenesis as efficiently as ACTH or colchicine although each has a different time course. These findings confirm the model that polymerized filaments can act as a barrier of cholesterol processing in steroid-producing cells.

. Drs. J. Wolff and D. Sackett, L. Knippling

Pure rat brain tubulin can be cross-linked by ultraviolet irradiation of tubulin-colchicine complexes at the high wavelength maximum of colchicine to form covalent dimers>trimers>tetramers. With colchicine concentrations $\sim 3 \times 10^{-4} M$ (mole ratio to tubulin 3-12) and irradiation for 5-10 min at 95-109 mW/cm², the yield of dimers is 11-17% and of trimers is 4-6% of the total tubulin. The oligomers show polydispersity and anomalously high apparent molecular masses that converge toward expected values in low-density gels. Maximal dimer yields are obtained with MTC and the decreasing photosensitizing potency is MTC>colchicine>colchicide>isocolchicine>thiocolchicine. Single-ring troponoids also promote dimerization. The initial, low-affinity, binding step of colchicine and its analogues is sufficient to photosensitize tubulin dimerization.

. Drs. J. Wolff, D. Sackett and S. Uppuluri and L. Knippling

Stepwise denaturation of pure tubulin with urea shows that the five readily measured function properties differ markedly in the sensitivity to different urea concentration and suggest stepwise unfolding of the molecule.

. Drs. J. Wolff, D. Sackett and S. Uppuluri and L. Knippling

VIII. PHYSICAL BIOCHEMISTRY

The concentration gradients of radiolabeled proteins at sedimentation equilibrium were measured with high accuracy and precision using a new microfractionation device and new counting techniques.

. Drs. S. Darawshe, G. Rivas, P. Jeffrey, and A. P. Minton

The calcium-linked self-association of human complement sub-component C1s was quantitatively characterized via measurement of sedimentation equilibrium.

. Drs. G. Rivas and A. P. Minton

A quantitative model for the role of macromolecular crowding in the regulation of cellular volume has been developed.

. Dr. A. P. Minton

A microprocessor-controlled, highly automated centrifuge tube microfractionator has been developed by BRANDEL, Inc. (Gaithersburg, MD) in collaboration with our group. Using this instrument, the contents of a small cylindrical centrifuge tube may be fractionated into laminar aliquots with a resolution as high as 10 fractions/mm of solution column, with minimal mixing between the contents of adjacent fractions. A new mode of fraction collection has been introduced which permits the collection of fractions directly on to scintillation vial caps impregnated with solid scintillator (Beckman Ready Caps), thus avoiding the use of scintillation fluid. Using this instrument we have measured the concentration gradients of a series of well-characterized radiolabeled proteins ranging in molecular weight from 45,000 to 350,000 centrifuged to sedimentation equilibrium. Our results indicate that molecular weights of non-associating macromolecules, and weight-average molecular weights of

associating macromolecules, may be rapidly, accurately, and conveniently measured using the new instrument, thus facilitating the study of reversible macromolecular self- and hetero-associations in solution. A novel application was the determination of the molecular weight of insulin at a concentration of 10 ng/ml, which is lower than previous concentrations at which this measurement was carried out by a factor of 10,000.

. Drs. S. Darawshe, G. Rivas, P. Jeffrey and A. P. Minton

The first component of the complement pathway, C1, is a noncovalent complex of five polypeptide chains, $\text{Clq} - (\text{Clr})_2 - (\text{Cls})_2$. Purified subcomponent C1s can exist as a monomer or dimer in solution. By means of sedimentation equilibrium we have measured the weight-average molecular weight of purified C1s in HEPES-NaCl buffer at 10°C as a function of protein and calcium concentration, over a range of total protein concentration exceeding two orders of magnitude and over a range of free (unbound) calcium concentration exceeding six orders of magnitude. The entire set of data comprised weight-average molecular weights corresponding to 85 different combinations of total protein and calcium concentrations, where each molecular weight was the mean of values obtained from 3 to 6 replicate experiments. A series of models for calcium-linked dimerization, in which different numbers and types of calcium binding sites on the monomer and dimer were postulated, were fit to the combined data by the method of nonlinear least squares. According to the simplest model capable of accommodating all of the data, monomeric C1s binds 1 Ca with an equilibrium association constant of $\sim 3 \times 10^{-5} \text{ M}^{-1}$, and dimeric C1s binding 1 Ca with high affinity ($K \sim 8 \times 10^{-7} \text{ M}^{-1}$) and two Ca with lower affinity ($K \sim 3 \times 10^{-4} \text{ M}^{-1}$). The high affinity site is presumed to lie at the interface between the polypeptide chains in the dimer.

. Drs. G. Rivas and A. P. Minton

In collaboration with Drs. J. C. Parker and G. C. Colclasure of the Department of Medicine, University of North Carolina at Chapel Hill, a simple model has been developed to account for large increases in transporter-mediated ion flux across cell membranes, that are elicited by small fractional changes of cell volume. The model is based upon the concept that, as a result of large excluded volume effects in cytoplasm (macromolecular crowding), the tendency of soluble macromolecules to associate with membrane proteins is such more sensitive to changes in cell water content than expected on the basis of simplistic considerations of mass action. The model postulates that an ion transporter may exist in either an active dephosphorylated state or an inactive phosphorylated state, and that the steady-state activity of the transporter reflects a balance between the rates of phosphatase-catalyzed activation and kinase-catalyzed inactivation. Cell swelling results in the inhibition of kinase relative to phosphatase activity, thereby increasing the steady-state concentration of the active form of the transporter. Calculated volume-dependent stimulation of ion flux is comparable to that observed experimentally.

. Dr. A. P. Minton

Cooperative binding systems are being studied taking into account site or subunit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems.

Amino acid sequences of proteins are analyzed primarily with the Monte Carlo techniques to evaluate the uniqueness and similarity of these sequences. The property of uniqueness (the occurrence of a *small* peptide at a frequency considerably less than that expected) has been quantified, and speculations on this quantity and the immune response are under continued investigation.

. Drs. H. A. Saroff and E. Mihalyi

Glutathione and another substance have been previously indicated to be regulators of the activity of a valyl-tRNA synthetase. The latest evidence is that the critical activator substance is an iron-containing polypeptide that affects the enzyme's affinity for tRNA.

. Dr. S. Black

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 23,330-14 LBP
PERIOD COVERED October 1, 1991, through September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Aldoheptose Biosynthesis and Its Regulation and Hepatitis Non-A, Non-B		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: William G. Coleman, Jr., Ph.D. Research Microbiologist LBP NIDDK		
Others: Lishi Chen, Ph.D. Visiting Associate LBP NIDDK Li Ding, Ph.D. Visiting Fellow LBP NIDDK		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Pharmacology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS: 3.8	PROFESSIONAL: 3.0	OTHER: 0.8
CHECK APPROPRIATE BOX(ES) * (a) Human subjects (b) Human tissues * x (c) Neither * (a1) Minors * (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Aldoheptose Biosynthesis. <i>E. coli</i> K-12 aldoheptose (i.e., L-glycero-D-mannoheptose) mutants carrying the <i>cysE-pyrE</i> linked mutation <i>rfaD</i> or <i>rfa-2</i> , were previously isolated and genetically characterized. These mutations result in L-glycero-D-mannoheptose deficient Lipopolysaccharide (LPS). Thus, the <i>rfaD</i> and <i>rfa-2</i> mutants produce a truncated LPS that is primarily a lipid A-2-keto-3-deoxyoctulosonic acid structure (i.e., chemotype Re). These mutants possess an outer membrane (OM) that is characteristically more permeable to a number of hydrophobic agents. The molecular genetics and biology of the <i>rfaD</i> gene has been completed and reported by this laboratory. An efficient <i>rfaD</i> gene expression system and a two-step purification protocol for the <i>rfaD</i> gene product (ADP-L-glycero-D-mannoheptose-6-epimerase) was developed. The enzyme thus purified was found to be greater than 95% pure, and it was highly active. Key kinetics and physical characterization studies of the epimerase are complete. Preliminary crystals of the epimerase have been obtained. Structural and functional homology of the <i>Pseudomonas aeruginosa rfaD</i> gene and its <i>E. coli</i> counterpart is indicated. The second mutation, designated <i>rfa-2</i> , has been exploited to define the <i>rfa-2</i> gene and resolve its sequence and flanking nucleotides, as well as determine its physical location on the <i>E. coli</i> chromosome relative to the <i>rfaD</i> gene. A <i>rfa-2</i> -6xHISTAG gene fusion has been constructed to facilitate the purification of the <i>rfa-2</i> gene product. Interspecific complementation studies demonstrated that the <i>E. coli</i> K-12 <i>rfa-2</i> gene complements the chemotype Re LPS mutant of <i>S. typhimurium</i> , designated <i>rfaC</i> . The role of the <i>rfa-2</i> gene product as a heptosyl transferase is under investigation.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 23,580-29 LBP
PERIOD COVERED October 1, 1991, through September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mammalian Transposons		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Anthony V. Furano, M.D. Medical Officer (Research) and Chief, Section on Genomic Structure and Function LBP NIDDK		
Others: Karen Usdin, Ph.D. Visiting Associate LBP NIDDK Bruce E. Hayward, Ph.D. Visiting Associate LBP NIDDK Mary Zavanelli, Ph.D. IRTA LBP NIDDK Renée Howell, Ph.D. IRTA LBP NIDDK		
COOPERATING UNITS (If any) Dr. Francois Catzeflis, Institute of Evolutionary Science, Montpellier, France; Dr. Michael Seidman, Otsuka Pharmaceuticals, Rockville, MD		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Genomic Structure and Function		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS: 4.3	PROFESSIONAL: 3.3	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) (a) Human subjects (b) Human tissues x (c) Neither (a1) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.) Members of the L1 transposon family (long interspersed repeat DNA or LINE family) of rats are 6.7 kb long, 5 kb of which is devoted to two protein encoding genes. A transcriptional regulatory region is at the left end of the element, and a guanine-rich polypurine:polypyrimidine sequence is near the right end. Although the protein encoding sequences of mammalian L1 families are highly conserved, the promoter sequences are completely distinct. This means that these families have been independently amplified in various mammalian species. Our recent studies of an ancient L1 family as well as recently evolved clades of the modern rat L1 family indicate that episodic L1 amplification has occurred repeatedly during mammalian evolution. We have also shown that analysis of an ancient amplification event can be a novel and powerful tool for determining phylogenetic relationships between modern animals. Our previous demonstration that the rat L1 family contains an active regulatory region was the first evidence that L1 DNA is not just some non-functional "junk" DNA. We have further characterized the L1 regulatory region and found that it contains three regions that can form specific DNA:protein complexes with nuclear proteins. Deletion analysis shows that one region binds a transcriptional stimulatory factor. We also have found that the L1 regulatory region activates both known and cryptic promoters located 5', but not 3', to it, in an orientation dependent manner. Therefore, the L1 regulatory region is not an "enhancer" sequence in the usual sense. We previously showed that the L1 guanine-rich polypurine:polypyrimidine sequence destabilizes contiguous duplex DNA, adopts several non-B DNA structures in vitro, and that the L1 non-B structures compete with target site non-B structures for supercoil energy which in vivo might modulate the supercoil dependent properties of L1 elements and their target sites. We now find that the L1 polypurine:polypyrimidine sequence decreases the replication of plasmids in both bacteria and mammalian cells and alters the apparent activity of certain eukaryotic promoters in vivo. In parallel studies we have shown that homoguanine stretches enhance the mutation and recombination rate of adjacent DNA sequences in vivo.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 23,750-06 LBP
PERIOD COVERED October 1, 1991, through September 30, 1992		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders) Bacteriophage T4 Gene Expression		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	Deborah M. Hinton, Ph.D.	Research Chemist LBP NIDDK
Others:	Mridula Sharma, Ph.D. Roslyn March-Amegadzie, Ph.D.	Visiting Fellow LBP NIDDK Senior Staff Fellow LBP NIDDK
COOPERATING UNITS (If any)		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Nucleic Acid Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS	PROFESSIONAL	OTHER
3.5	3.0	0.5
CHECK APPROPRIATE BOXES		
(a) Human subjects (b) Human tissues X (c) Neither (a1) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type Do not exceed the space provided)		
<p>During T4 infection, transcriptional control is accomplished by phage-encoded factors that alter the specificity of the host RNA polymerase. Using an <i>in vitro</i> transcription system composed of T4-modified host RNA polymerase and the T4 transcriptional activator MotA, we are investigating the action of MotA at the T4 middle promoter PuvSX. We have found that transcription from PuvSX <i>in vitro</i> is sensitive to the addition of the polyanion heparin before but not after the addition of MotA. This result suggests that MotA is needed to form an open complex at PuvSX (the complex in which polymerase has partially unwound the promoter) since open complexes are known to be heparin resistant. We have partially purified and characterized a mutant MotA protein, MotA21 in which the first 8 amino acids of MotA have been substituted with 11 different amino acids. Although MotA21 binds to the PuvSX promoter nearly as well as MotA, MotA21 does not support transcription from PuvSX <i>in vitro</i>. These results are consistent with the motA21 mutation affecting a domain of MotA that is involved in transcriptional activation rather than DNA binding.</p> <p>The T4 gene <i>segA</i> encodes a protein that shares regions of similarity with a family of group I intron-encoded endonucleases present in fungi and phage. These proteins are required for the movement ('homing') of the intron DNA into its intronless gene, cutting at or near the site of intron insertion. Like these endonucleases, SegA is a Mg⁺⁺-dependent DNA endonuclease which cuts with some sequence specificity, but it does not appear to be associated with an intron. We have used primer extension analyses to determine the exact positions of cutting by SegA at three preferred sites. While these three sites share some sequence elements, a consensus sequence cannot be deduced. This suggests that either SegA does not require a set of invariant bases or it recognizes some feature of the DNA other than sequence alone. From a screening of the genomes of 30 T-even like phage, we find that the <i>segA</i> gene appears to be missing in all these genomes, consistent with the idea that the <i>segA</i> gene was or is mobile.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 23,900-01 LBP
PERIOD COVERED October 1, 1991, through September 30, 1992		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders) Chemistry and Function of Microtubules		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Jan Wolff, M.D., Ph.D. Medical Officer and Chief, Section on Endocrine Biochemistry	LBP NIDDK
Others:	Dan Sackett, Ph.D. Expert Shobha Uppuluri, Ph.D. Visiting Associate Leslie Knippling Biologist	LBP NIDDK LBP NIDDK LBP NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH		
Laboratory of Biochemical Pharmacology		
SECTION		
Section on Endocrine Biochemistry		
INSTITUTE AND LOCATION		
NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS	PROFESSIONAL	OTHER
4.2	3.0	1.2
CHECK APPROPRIATE BOXES		
(a) Human subjects (b) Human tissues X (c) Neither (a1) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)		
<p>Pure rat brain tubulin can be cross-linked by ultraviolet irradiation of tubulin-colchicine complexes at the high wavelength maximum of colchicine to form covalent dimers>trimers>tetramers. With colchicine concentrations $\sim 3 \times 10^{-4} \text{M}$ (mole ratio to tubulin 3-12) and irradiation for 5-10 min at $95-109 \text{ mW/cm}^2$, the yield of dimers is 11-17% and of trimers is 4-6% of the total tubulin. The oligomers show polydispersity and anomalously high apparent molecular masses that converge toward expected values in low-density gels. Maximal dimer yields are obtained with MTC and the decreasing photosensitizing potency is MTC>colchicine>colchicide>isocolchicine>thiocolchicine. Single-ring troponoids also promote dimerization. The initial, low-affinity, binding step of colchicine and its analogues is sufficient to photosensitize tubulin dimerization.</p> <p>Stepwise denaturation of pure tubulin with urea shows that the five readily measured function properties differ markedly in the sensitivity to different urea concentration and suggest stepwise unfolding of the molecule.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 24,140-26 LBP
PERIOD COVERED October 1, 1991, through September 30, 1992		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders) Structure and Function of the Tryptophan Synthase Multienzyme Complex		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) PI: Edith Wilson Miles, Ph.D. Research Chemist and Chief, Section on Enzyme Structure and Function LBP NIDDK		
Others: Syed A. Ahmed, Ph.D. Senior Staff Fellow LBP NIDDK Xiang-Jiao Yang, Ph.D. Visiting Fellow LBP NIDDK Sergei Ruvinov, Ph.D. Visiting Fellow LBP NIDDK Zichun Lu, Ph.D. Visiting Fellow LBP NIDDK		
COOPERATING UNITS (If any) Drs. D.R. Davies, C.C. Hyde & K. Parrish, LMB, NIDDK; P. McPhie, LMB, NIDDK; A. Ginsburg & D.P. Raneta, LB, NHLBI; P. Brzovic & M.F. Dunn, Univ. of California, Riverside; A. Mozzarelli & G.L. Rossi, Univ. of Parma, Italy; K. Yutani, Osaka U., Japan; K.S. Anderson, Yale Univ., New Haven, CT; & K.A. Johnson, Pennsylvania State Univ., University Park, PA		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Enzyme Structure and Function		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS 4.9	PROFESSIONAL 4.4	OTHER 0.5
CHECK APPROPRIATE BOXES (a) Human subjects (b) Human tissues X (c) Neither (a1) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided) The tryptophan synthase $\alpha_2\beta_2$ multienzyme complex is an excellent model system for investigating enzyme mechanism, protein-protein interaction, the allosteric mechanism and metabolite channeling. Correlations between the functional properties of wild type and mutant forms of tryptophan synthase and the three-dimensional structure have produced recent important results: (1) <u>Mechanism of pyridoxal phosphate-dependent reactions.</u> Lysine-87 forms a Schiff base with the pyridoxal phosphate coenzyme in active site of the wild type β subunit. Changing lysine-87 to threonine produces an inactive mutant $\alpha_2\beta_2$ complex that forms enzyme-substrate intermediates very slowly. Spectroscopic and kinetic studies of this mutant enzyme show that lysine-87 serves critical roles in transamination, catalysis, and product release. Two new crystallographic structures of this mutant enzyme containing bound L-serine or L-tryptophan reveal important information about the substrate binding site of the β subunit. (2) <u>Mechanism of subunit communication.</u> Studies using site-directed mutagenesis and limited proteolysis provide evidence that a flexible loop in the α subunit is important for ligand binding and for communicating the effects of ligand binding from the α subunit to the β subunit in the $\alpha_2\beta_2$ complex. A residue in the α subunit loop (threonine-183) plays a critical role in modulating the enzymatic activity of the β subunit in the $\alpha_2\beta_2$ complex. Two α subunit residues that are located in the interaction site between the α and β subunits (proline-57 and proline-132) are important for mutual subunit interaction and activation. Mutation of glutamate-49, aspartate-60, or glycine-51 in the α subunit inhibits the transition of the α subunit from open to a closed form and alters the kinetics of metabolite channeling. (3) <u>Conformational states of the β subunit.</u> Our finding that the dimeric wild type β subunit is only 50% inactivated by β -chloro-L-alanine can be explained by the presence of two conformers of the subunit in solution: one conformer is rapidly inactivated by β -chloro-L-alanine and the other conformer is not activated. This putative mechanism is supported by investigations using steady-state kinetics and spectroscopic and electrophoretic methods.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 24,150-21 LBP
PERIOD COVERED October 1, 1992, through September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders) Noncovalent intermolecular interactions in Biochemistry		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Allen P. Minton, Ph.D. Research Chemist and Chief, Section on Physical Biochemistry LBP NIDDK		
Others: German Rivas, Ph.D. Visiting Fellow LBP NIDDK Saleh Darawshe, Ph.D. Visiting Fellow LBP NIDDK Peter Jeffrey, Ph.D. Courtesy Associate LBP NIDDK		
COOPERATING UNITS (If any) J. Bertocini, Biomedical Research & Development Lab., Inc., Gaithersburg, MD; J. C. Parker, M.D., Dept. of Medicine, University of North Carolina at Chapel Hill, NC		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Physical Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS 3.4	PROFESSIONAL 3.2	OTHER 0.2
CHECK APPROPRIATE BOXES (a) Human subjects (b) Human tissues x (c) Neither (a1) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The concentration gradients of radiolabeled proteins at sedimentation equilibrium were measured with high accuracy and precision using a new microfractionation device and new counting techniques.</p> <p>The calcium-linked self-association of human complement sub-component C1s was quantitatively characterized via measurement of sedimentation equilibrium.</p> <p>A quantitative model for the role of macromolecular crowding in the regulation of cellular volume has been developed.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 24,260-26 LBP
PERIOD COVERED October 1, 1991 through September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Enzymatic Mechanisms of DNA Replication: The Bacteriophage T4 System		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI: Others:	Nancy G. Nossal Research Chemist and Chief, Section on Nucleic Acid Biochemistry Lisa Hobbs, Ph.D IRTA Fellow Todd Capson, Ph.D Senior Staff Fellow	LBP NIDDK LBP NIDDK LBP NIDDK
COOPERATING UNITS (if any) Dr. Craig Hyde, LSBR NIAMS Dr. Stephen Benkovic, Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Nucleic Acid Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS 3.6	PROFESSIONAL 3.0	OTHER 0.6
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) We are continuing our study of the <i>E. Coli</i> bacteriophage T4 model system for duplex DNA replication in which efficient DNA replication <i>in vitro</i> is achieved with purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43), gene 32 DNA helix-destabilizing protein, the gene 44/62 and gene 45 polymerase accessory proteins, the genes 41, 61, and 59 primase-helicase, RNase H, and DNA ligase. <i>Assembly of the polymerase and accessory proteins on the primer template.</i> We have previously used cross-linking of proteins to photo-activatable residues at specific nucleotides in the primer to determine the position of polymerase and each of the accessory proteins on the primer. In order to position these proteins on the template strand, and to study the steps in the assembly of the complex by a gel-mobility shift assay, we are conducting a systematic analysis to determine the minimum size primer-template that will allow the proper assembly of the complex. <i>Construction and analysis of mutations in T4 DNA polymerase.</i> T4 DNA polymerase has extensive amino acid sequence similarity with a large family of prokaryotic and eukaryotic DNA polymerases. We are using <i>in vitro</i> mutagenesis to determine the function of these conserved regions and to try to identify regions of the polymerase important for its interaction with the other T4 replication proteins. We have constructed a polymerase lacking the editing exonuclease by changing a conserved amino acid in the N-terminal region. This mutant retains its polymerase activity and interacts normally with the accessory proteins, T4 phage with the exonuclease defective polymerase gene have a very high rate of mutation <i>in vivo</i> . <i>Function of the T4 gene 59 helicase assembly factor.</i> The addition of 59 protein to the other seven replication proteins gives a marked increase in synthesis on duplex DNA. We are currently studying how and where this synthesis is initiated. T4 RNaseH. We have identified a T4 gene encoding an RNaseH, and shown that the purified protein removes RNA primers <i>in vitro</i> . We have constructed a phage mutant with a deletion within the RNaseH gene, and shown that this gene is essential in a host that is lacking both RNaseH and 5' to 3' exonuclease of Pol I. <i>Structure of the T4 replication proteins.</i> We have begun a collaboration with Craig Hyde, NIAMS, to try to crystallize these proteins.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 24,590-22 LBP
PERIOD COVERED October 1, 1991, through September 30, 1992		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) Structure and Interactions of Biologically Important Macromolecules		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	Harry A. Saroff, Ph.D. Research Chemist (Intermittent)	LBP NIDDK
Other:	Elemer Mihalyi, M.D., Ph.D. Special Volunteer	LBP NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Pharmacology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.7	1.5	0.2
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)		
<p>Cooperative binding systems are being studied taking into account site or sub-unit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems.</p> <p>Amino acid sequences of proteins are analyzed primarily with the Monte Carlo techniques to evaluate the uniqueness and similarity of these sequences. The property of uniqueness (the occurrence of a small peptide at a frequency considerably less than that expected) has been quantified, and speculations on this quantity and the immune response are under continued investigation.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 24,709-11 LBP						
PERIOD COVERED October 1, 1991, through September 30, 1992								
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Polyamine Biosynthesis and Function								
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;"> PI: Celia White Tabor, M.D. </td> <td style="width: 33%; vertical-align: top;"> Medical Officer (Research) </td> <td style="width: 33%; vertical-align: top;"> LBP NIDDK </td> </tr> <tr> <td colspan="3" style="padding-top: 10px;"> Others: Herbert Tabor, M.D. Supervisory Medical Officer (Research) Chief, Section on Pharmacology, LBP; and Chief, Laboratory of Biochemical Pharmacology LBP NIDDK David Balasundaram, Ph.D. Visiting Associate LBP NIDDK Nobuko Hamasaki, Ph.D. IRTA LBP NIDDK </td> </tr> </table>			PI: Celia White Tabor, M.D.	Medical Officer (Research)	LBP NIDDK	Others: Herbert Tabor, M.D. Supervisory Medical Officer (Research) Chief, Section on Pharmacology, LBP; and Chief, Laboratory of Biochemical Pharmacology LBP NIDDK David Balasundaram, Ph.D. Visiting Associate LBP NIDDK Nobuko Hamasaki, Ph.D. IRTA LBP NIDDK		
PI: Celia White Tabor, M.D.	Medical Officer (Research)	LBP NIDDK						
Others: Herbert Tabor, M.D. Supervisory Medical Officer (Research) Chief, Section on Pharmacology, LBP; and Chief, Laboratory of Biochemical Pharmacology LBP NIDDK David Balasundaram, Ph.D. Visiting Associate LBP NIDDK Nobuko Hamasaki, Ph.D. IRTA LBP NIDDK								
COOPERATING UNITS (If any)								
LAB/BRANCH Laboratory of Biochemical Pharmacology								
SECTION Section on Pharmacology								
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892								
TOTAL STAFF YEARS: 4.8	PROFESSIONAL: 3.6	OTHER: 1.2						
CHECK APPROPRIATE BOX(ES) * (a) Human subjects (b) Human tissues * x (c) Neither * (a1) Minors * (a2) Interviews								
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Polyamines (putrescine, spermidine, and spermine) are major cellular components and have been shown to be involved in many systems related to growth and differentiation. Our current and older studies have been directed at learning how these polyamines are synthesized and regulated, and their physiological function. We have: (1) established the pathways for the biosynthesis of these amines in prokaryotes and eukaryotes and isolated the enzymes for the various steps in the pathways; (2) identified the genes responsible for each of the biosynthetic steps and constructed mutants with deletions in the various genes; (3) constructed plasmids that contain these genes and used the strains containing these plasmids to overproduce the encoded enzymes; (4) used the amine-deficient mutants to study the physiological effects of polyamine deprivation; (5) sequenced the gene coding for S-adenosylmethionine decarboxylase in both <i>E. coli</i> and <i>S. cerevisiae</i> and the gene coding for spermidine synthase in <i>E. coli</i>; (6) demonstrated that S-adenosylmethionine decarboxylase is first formed as a proenzyme in both <i>E. coli</i> and yeast and is cleaved post-translationally with the conversion of serine to a covalently-bound pyruvoyl group that is essential for activity; and (7) studied the effect of site-specific mutagenesis on the conversion of the proenzyme to the active enzyme. The most recent work involves the physiological effects of complete polyamine deprivation resulting from a null mutation in the gene (<i>spe2</i>) for S-adenosylmethionine decarboxylase in <i>S. cerevisiae</i>. In addition to marked microscopic changes, these polyamine-deficient cells show gross abnormalities in the cell envelope and in the mitochondria, indicating the importance of spermidine/spermine in the biosynthesis and/or integrity of these cell moieties. Many of our recent studies suggest that polyamines have a critical role in the protection of the yeast cell against oxidative damage <i>in vivo</i>. </p>								

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 24,940-19 LBP
PERIOD COVERED October 1, 1991, through September 30, 1992		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders) Yeast RNA Virology		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	Reed B. Wickner, M.D.	Medical Officer, USPHS
	and Chief, Section on Genetics of Simple Eukaryotes	LBP NIDDK
Others:	Tsutomu Fujimura, Ph.D.	Visiting Scientist
	Yutaka Matsumoto, Ph.D.	Staff Fellow
	Jonathan D. Dinman, Ph.D.	Staff Fellow
	William R. Widner, Ph.D.	IRTA Fellow
	Rosaura P. C. Valle, Ph.D.	Visiting Fellow
	Juan C. Tercero Lopez, Ph.D.	Special Volunteer
	Juan Carlos Ribas, Ph.D.	Special Volunteer
COOPERATING UNITS (if any)		
LAB/BRANCH		
Laboratory of Biochemical Pharmacology		
SECTION		
Section on Genetics of Simple Eukaryotes		
INSTITUTE AND LOCATION		
NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS	PROFESSIONAL	OTHER
7.6	7.2	0.4
CHECK APPROPRIATE BOX(ES)		
(a) Human subjects (b) Human tissues X (c) Neither (a1) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)		
<p>We study the mechanism of propagation of the L-A dsRNA virus of the yeast <i>Saccharomyces cerevisiae</i>, the host genes which it uses for this purpose and the mechanisms by which the host limits viral propagation to prevent viral cytopathology. We find that the N-terminal 1/4 of the L-A pol gene is needed for encapsidation of viral RNA, but that only gag is needed to assemble viral particles. We have found that pol encodes three ssRNA binding regions, one of which is cryptic, its activity being inhibited by a region C-terminal to this binding region. One of the ssRNA binding regions of pol is within the region necessary for packaging, suggesting that this is its <i>in vivo</i> role. The others are likely to be involved in RNA replication and transcription. The assembly process requires the <u>MAK3</u> N-acetyltransferase that acetylates the N-terminus of gag. Stable particle formation also requires the <u>MAK10</u> protein, particularly if the particles are completely full.</p> <p>The six <u>SK1</u> genes comprise a host system to limit the replication of three unrelated dsRNA and ssRNA replicons. We have obtained evidence that they do this by limiting the translation of uncapped messages.</p> <p>We have isolated mutations in several chromosomal genes that result in substantial increases in the efficiency of the -1 ribosomal frameshifting that L-A uses to synthesize its gag-pol fusion protein. We find that the efficiency of frameshifting is critical to viral propagation, suggesting that drugs affecting this process may be useful against retroviruses.</p> <p>The [URE3] non-Mendelian genetic element of yeast requires for its propagation the <u>URE2</u> chromosomal gene. We have evidence that [URE3] is a prion of yeast.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 24,941-01 LBP
PERIOD COVERED October 1, 1991, through September 30, 1992		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) Membranes Cytoskeleton and Secretion		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	Jan Wolff, M.D., Ph.D. and Chief, Section on Endocrine Biochemistry	Medical Officer LBP NIDDK
Others:	Dan Sackett, Ph.D. Leslie Knipping	Expert Biologist LBP NIDDK LBP NIDDK
COOPERATING UNITS (if any) T. Shiver, NICHD		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Endocrine Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS 3.2	PROFESSIONAL 2.0	OTHER 1.2
CHECK APPROPRIATE BOX(ES) (a) Human subjects (b) Human tissues <input checked="" type="checkbox"/> (c) Neither (a1) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)		
<p>The complex role of the cytoskeleton in the intracellular traffic of cholesterol from droplet to mitochondrion involves not only microfilaments and microtubules (as we have previously shown), but also involves the participation of intermediate filaments, as we now show for the first time. The disruption of intermediate filaments with acrylamide enhances steroidogenesis as efficiently as ACTH or colchicine although each has a different time course. These findings confirm the model that polymerized filaments can act as a barrier of cholesterol processing in steroid-producing cells.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 24942-16 LBP

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Adenylate Cyclase and Other Extracellular Products of B. Pertussis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Wolff Associate Chief CEB, NIDDK

Others: D. Sackett Expert CEB, NIDDK

L. Knipling Technician CEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Endocrine Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

The project is inactive.

Annual Reports of the Laboratory of Chemical Biology National Institute of Diabetes and Digestive and Kidney Diseases

The Laboratory of Chemical Biology conducts research on molecular biology and genetics, especially as related to genetic diseases, and on the structure, function and dynamics of proteins. A major emphasis of the Laboratory is in understanding the molecular processes involved in the developmental control of the expression of the human hemoglobin genes. As part of this work, extensive studies on the pathophysiology of genetic diseases of hemoglobin and new approaches to their treatment have been developed. A second major emphasis of the Laboratory is in the study of forces that stabilize globular proteins. A third program concentrates on cytogenetic analysis of patients with genetic illnesses. Other new research initiatives include the study of transcriptional control of globin genes by silencers and the cloning and characterization of the human erythropoietin receptor gene. The protocol for treatment of sickle cell disease patients with hydroxyurea to elevate fetal hemoglobin has been enlarged to include protocols with the addition of recombinant human erythropoietin and also the treatment of patients with the thalassemia syndromes. A facility for the production of transgenic mice has been established.

The Laboratory has three Sections. The Section of Protein Chemistry and Conformation, under Dr. Hiroshi Taniuchi, is devoted primarily to studying the folding and assembly of globular proteins, especially cytochrome c. The Section on Molecular Forces and Assembly is the home of the Cytogenetics Unit under Dr. Beverly White, which is a joint endeavor of the Inter-Institute Genetics Program of the Clinical Center and NIDDK. The Section on Molecular Biology and Genetics, under Dr. Alan N. Schechter, is concerned primarily with the molecular basis of the developmental control of gene expression, especially in human erythroid cells, and its relevance to the understanding of the molecular basis of genetic diseases of hemoglobin and the red blood cell and approaches to their therapy. A Unit on Molecular Hematology, under Dr. Griffin Rodgers, has been established in that Section.

During this last year, several personnel changes have occurred. Dr. Griffin P. Rodgers has been appointed head of the new Unit on Molecular Hematology. Dr. Betty Peters is the recipient of a fellowship from the Robert Wood Johnson Foundation Minority Medical Faculty Development Program. Dr. Yongji Wu, of the Peking Union Medical College in Beijing, has worked in the Laboratory for an extended period under the Courtesy Associate Program for senior scientific visitors. Dr. Eitan Fibach of the Hadassah Medical School in Jerusalem Israel has come to the Laboratory as a Visiting Associate. Dr. Suthat Fucharon of the Maidol Medical School in Bangkok, Thailand have worked in this Laboratory under sponsorship of the World Health Organization. Dr. Byung-kook Kim of Seoul National University School of Medicine has been a Visiting Scientist in this Laboratory under sponsorship of the Korean government. Many students, especially those in the MARC/MBSR Program, have spent periods of 2 to 3 months during the summer in this laboratory. Dr. C. B. Anfinsen continues as a Scientist Emeritus in this Laboratory and is a frequent visitor.

Extensive research collaborations exist within this Laboratory and with other Laboratories in this Institute, in NIH, and nationally and internationally as outlined in the individual Research Project Reports. A formal collaboration has been established with the

Clinical Center's Inter-Institute Medical Genetics Program to fund a clinical and research cytogenetic program. Clinical collaborations also exist with the Clinical Hematology Branch of NHLBI and other units for the purpose of evaluating new therapies for sickle cell disease and the thalassemia syndromes. Collaborations with several industrial firms, including Isolabs, Inc. of Akron, Ohio and Chi Associates of Alexandria, Virginia are being established by various new administrative mechanisms.

Section on Protein Chemistry and Conformation

There are two major projects underway in this Section, both with the goal of predicting the three dimensional structure of proteins on the basis of the amino acid sequence. One project uses the fragment complex system of cytochrome c for studies of the kinetics of folding and unfolding and the thermodynamics and the structural properties of these models for studying protein folding. Over the years several different complexes of cytochrome c fragments have been characterized and the data has led to a model which has recently been published. This model describes folding in terms of four core domains. Each core domain consists of a hydrophobic core and its surrounding shell and folds and unfolds as a unit. Analysis of the free energy change for folding and the heat stability of various complexes containing substituted amino acids suggests that folding is associated with an increase in the extent of the core group interaction. This interaction is apparently of long range and does not seem to involve solvent. It is hypothesized that folding of one complex, which resembles native cytochrome c, is a process of unification of the ordered core of the core domains and that this unification is associated with expansion of the core group interaction to generate extra stabilizing energy. This hypothetical core group interaction is assumed to represent a new type of non-covalent interaction.

A second project is to study antigen-binding interactions, which are fundamental for immunology. While many consider a combination of hydrophobic interactions, van der Waal interactions, hydrogen bonds and electrostatic interactions as sufficient to describe this interaction, this project asks whether long-range core group interactions exists to stabilize the structure. For this study, amide hydrogen-exchange of three monoclonal IgG antibodies to yeast iso-1-cytochrome c and the Fab fragment of one of these antibodies in the presence and absence of the immunogen and evolutionarily related species has been measured. The number of hydrogens of the monoclonal antibodies whose exchange was suppressed on binding to the immunogen was found to exceed that estimated for the residues shielded by the immunogen. Suppression occurs mainly for the Fab domains, but not for the Fc. One of the antibodies showed two distinct classes of amide-hydrogens. Class 1 hydrogens exchange faster than class 2. The exchange of class 1 hydrogens was suppressed by binding to the immunogen but not to the evolutionarily related species. The exchange of class 2 hydrogens was suppressed by binding to the evolutionarily related species as well as to the immunogen. Thus, the suppression of exchange of class 1 hydrogens appears to occur by some kind of conformational stabilization, the mechanism of which differentiates between the immunogen and the evolutionarily related species. Evidence suggests that the trans-interactions of the Fab domain may modulate the hydrogen exchange. If it is assumed that the antigen-binding strengthens the trans-interactions in such a way that the exchange of the slower exchanging hydrogens is suppressed, this could explain the suppression of exchange of class 2 hydrogens. The studies are consistent with the idea that the antigen-antibody interaction is not confined to the antigen-antibody interface and that it may involve some long-range interaction with distant residues of the Fab. As a step to achieve mapping of such residues, the variable domain of the heavy

chain of one of the monoclonal antibodies has been essentially completely sequenced based on cDNA.

Section on Molecular Forces and Assembly

The Cytogenetics Unit reported cytogenetic analyses of 221 patients in Clinical Center protocols. Clinical studies of several endocrine disorders required confirmation of cytogenetic diagnosis and detection of genetic variation possibly affecting therapeutic response. Cytogenetic screening was also done to determine protocol eligibility or to reveal constitutional aberrations which might reveal gene localization in unmapped single gene disorders. In some diseases, secondary aberrations which might play a role in development of clinical abnormalities were analyzed. No cytogenetic abnormalities were seen in 29 new patients with mental disorders. However, a translocation previously detected in a schizophrenic patient was found in his relatives; cell lines were established from the family, but linkage studies were deferred because the rearrangement and mental problems were not consistently associated. Fluorescent in-situ hybridization (FISH) with chromosome-specific satellite DNA probes was used for investigation of a patient with premature ovarian failure and an X-autosome rearrangement, twin sisters with the Turner syndrome and a minute Y chromosome, a previously reported patient with mosaic Down syndrome, and a 46,XX male with hermaphroditism. Cell lines from several of these patients are being studied to detect and localize molecular defects and correlate them with clinical manifestations.

The Unit is affiliated with the NIH medical genetics program, is accredited by the ABMG to provide post-doctoral training in clinical laboratory cytogenetics, and provides clinical consultations. It also participates in the College of American Pathologists cytogenetics proficiency testing program, which is required for accreditation of the genetics and pathology training programs.

Section on Molecular and Biology and Genetics

The Section was involved in four major research areas: 1) Study of the molecular genetic control of the developmental biology of the human hemoglobin genes, especially the role of the epsilon globin gene transcriptional silencer; 2) Evaluation of the effects of hydroxyurea and erythropoietin on the treatment of genetic diseases of hemoglobin (sickle cell disease and thalassemia syndromes) and study of the genetic mechanism of action of these drugs; 3) Investigation of the pathophysiology of sickle cell disease and the development of methods to predict the severity of the disease and evaluate responses to therapy; and 4) Study of the transcriptional control of the human erythropoietin receptor and its role in the cell biology of human erythropoiesis. A brief summary of each of these areas of laboratory and clinical investigation follows:

1) Developmental Biology of Human Hemoglobin Genes

We have been studying the molecular genetic mechanisms that control the developmental switch from embryonic to fetal to adult hemoglobins with respect to their basic biology and their relevance to developing new therapies for diseases of hemoglobin. To study these processes we have been using two model systems: the K562 erythroleukemic cells and transgenic mice. K562 is an erythroleukemic cell line used for the last decade as a model for the study of the control of human globin gene expression. These cells do not support transcription of the beta-globin gene but do express transcripts

of epsilon- and gamma-globin genes at very high levels when exposed to a number of inducing agents. Results from this and other laboratories suggest that the control of this pattern of expression is mediated by the presence and/or absence of trans-acting factors which exert their action on sequences corresponding to the promoters of these genes. In the last few years transgenic mice have become an excellent model system for studying globin gene expression, and are supplementing the use of human cell lines.

We have previously reported the presence of a transcriptional control element with properties of a silencer extending from -392 to -177 bp relative to the cap site of the human epsilon-globin gene. Using deletion mutants and synthetic oligonucleotides in transient expression assays, DNA sequences responsible for this effect have been further delimited to 44 nucleotides located between -294 and -251 bp. Gel electrophoresis mobility shift assays and DNaseI footprinting assays demonstrate that these negative regulatory sequences are recognized differently by proteins present in nuclear extracts obtained from HeLa and K562 cells. The protein present in K562 cells, but not in HeLa cells, that interacts specifically with this silencer binds to the same sequence recognized by the yeast binding protein ABF1.

We have now used the K562 *in vitro* transcription system to examine the silencer. We find that K562 NE has markedly reduced synthesis of RNA *in vitro* from epsilon-globin gene DNA deletion templates which contain the silencer sequence, or part thereof, but not the adjacent 5' positive regulatory region (-453 to -535 bp). Furthermore, those transcripts generated *in vitro* from DNA templates extending to -453 bp or less of the epsilon-globin gene were not correctly initiated at the canonical cap site. Separating the K562 NE by ion exchange chromatography, we isolated fraction (F50) which contains the trans-acting factors associated with the silencer activity. This suppression by F50 was not observed on transcriptional activity of the permissive adenovirus 2 major late promoter. In electrophoretic mobility shift assays using the epsilon-globin gene silencer region as probe, F50 and F175 exhibited different DNA binding protein patterns: a specific protein band in F50 appears to be associated with the silencer activity.

Using DNase I footprinting, we have identified several sites of protein binding to the silencer. The major protected region shares a high percentage of homology with the binding sites of an erythroid specific transcription factor GATA-1, a yeast silencer binding protein, ABF-1, and the newly discovered YY1 transcriptional regulator. Using gel mobility shift assays with K562 nuclear extract, yeast ABF-1 protein and a probe bracketing the major protected region and competitor DNA with mutations in the sites homologous to GATA-1 and ABF-1 binding sites we have demonstrated that a protein, likely GATA-1, binds to the GATA site in the silencer and that another protein, likely a human homologue of ABF-1 or YY1, binds to the silencer. We are currently in the process of examining the function of mutations made in ABF-1/YY1 and GATA binding sites.

To delineate the molecular mechanism of the epsilon globin gene silencer activity and its possible role in the developmental regulation of epsilon gene expression, further characterization of epsilon gene transcription initiation sites in cell systems where the epsilon gene is up and down regulated, such as MB-02 and the two-phase liquid culture system of Fibach, was studied. A variety of clues in the literature and in our own data suggested that the epsilon silencer could affect transcription initiation. We have now shown that in MB-02 cells levels of correctly initiated epsilon transcripts are significantly lower than in K562 cells. Correctly initiated epsilon transcripts were not detectable in

normal cells prepared in the liquid culture system. We have, however, observed a smaller band in T1 analysis using a specific probe for exon I and 5' upstream region of the epsilon globin gene. Moreover, using the exon II of the epsilon gene as probe in T1 analysis we have detected the expected band and also a few smaller bands. Quantitating of epsilon exon I transcripts using cDNA amplification showed a small amount of correctly initiated epsilon transcripts in normal cells. Further characterization of the smaller bands will test the possible role of the epsilon gene silencer in regulation of developmental expression of the epsilon gene. The data so far are compatible with our original hypothesis that the silencer affects transcriptional initiation.

We have also initiated studies using the transgenic technique to study the epsilon-globin silencer; constructs with the silencer sequence mutated are being compared to those with these nucleotides intact for their effect on expression of the epsilon globin gene in the embryonic and adult mouse.

2) Treatment of Genetic Diseases of Hemoglobin

Our group has focused on pharmacologic manipulation of fetal hemoglobin levels in patients with genetic defects of hemoglobin. We have previously shown that hydroxyurea (HU) treatment of severely affected sickle cell patients results in about a 70 to 75% response rate defined. We have subsequently treated six patients with beta thalassemia intermedia with HU. Two patients showed an increase of at least 2-fold in HbF levels and of 5-fold in gamma globin mRNA. Curiously, two other patients showed an obvious increase primarily in beta-globin biosynthesis corresponding to an increase in beta-mRNA transcripts without a change in gamma globin synthesis. Thus, in addition to its effects in stimulating gamma globin synthesis, HU may be useful in the context of treatment of beta thalassemia through other mechanisms.

In an effort to achieve higher levels of hemoglobin F, in a more pancellular distribution, we have recently treated four patients with sickle cell disease who were receiving hydroxyurea for periods of 5-15 months on four consecutive days with escalating dose of recombinant erythropoietin (EPO) for 7 weeks, given on the alternate three days along with oral iron sulfate. In these four patients such combination therapy was associated with a 1.4 to 3 fold increase in F-reticulocytes, a 1.3 to 2 fold increase in F-cells and a 1.4 to 2 fold increase in the percentage of hemoglobin F when compared to the maximal values previously achieved on hydroxyurea alone.

In order to improve our understanding of the mechanisms of action of HU, two HbSS patient and two beta-thalassemia patient (with IVS-2 nt 654 cytosine to thymine, CD 41-42 mutations) who received HU treatment were studied for changes in alpha, beta, and gamma mRNA levels by our newly developed competitive polymerase chain reaction (PCR) method. PCR quantitation was achieved by reverse transcriptase (RT) production of cDNA from mRNA in nucleated peripheral blood cells and subsequent competitive analysis of absolute mRNA levels. The results showed that the gamma-globin mRNA amount was increased by about 50-fold and the absolute magnitude of the change in gamma-mRNA levels were similar in the SS and beta-thalassemia patients. Increases, but to a smaller extent, were also observed in alpha and beta globin mRNA levels. These results suggest that the molecular mechanism of HU (and perhaps other related compounds) is a general one on expression of all globin genes, but greatest for gamma.

In order to gain insight into the possible genetic mechanisms underlying the effects of HU, we used cation exchange (CE) and reverse-phase high performance liquid chromatography (HPLC) to examine changes in the levels of each hemoglobin species. Samples from ten patients treated with HU were analyzed approximately twice each week. Within the first 50 days of HU treatment, HbF increased on average from 3.3% to 7%. The patients had an average initial ratio of G-gamma/A-gamma of 0.72 which did not change with treatment. At baseline, approximately 15-20% of the fetal hemoglobin exists in the acetylated form (F1) as determined by CE-HPLC, independent of subtype. With HU treatment, the total amount of F0 and F1 increases roughly proportional to the increase in F-reticulocyte numbers. Although the total HbA2 level appears unaffected by treatment, there is an increase in the proportion of one modified HbS species, which may co-migrate with Hb A2, after about 50 days of treatment. For three patients treated with a combination HU and erythropoietin, we found a further increase in both F1 and this modified HbS fraction to levels approaching 5% and 10% respectively. Subsequent analysis of this modified beta-globin polypeptide showed that the NH2 was not blocked (Edman sequencing) and that the modifier had a mass of 300, close to the one of glutathione (307). A dual strategy using deblocking and synthetic methods has been used followed by IEF analysis and confirmed the glutathione nature of the adduct. Chemically modified HbS may interfere with HbS polymerization and thus have an added clinical benefit. Acetylation of other proteins (e.g. histones) may be involved in the regulation of gene expression; naturally occurring glutathione adducts have only been reported very recently. It is possible that their formation is related to metabolic effects of HU treatment or, as with acetylation, this modification may be indicative of induced fetal erythropoiesis.

The K562 human erythroleukemia cell line can be reversibly induced to increase gamma-globin gene expression in response to hydroxyurea and other agents. We are therefore using the K562 cell as a model system to understand the mechanism of induction of globin gene transcription. K 562 cells have been grown in the presence of 25mM hemin and 100mM hydroxyurea. We have used gel-retardation electrophoresis, DNA-footprinting (DNase I and methylation protection), *in vivo* genomic DNA footprinting, and ion exchange and affinity chromatography to investigate the interaction of both specific and non-specific proteins to globin gene promoter DNA. By gel shift analysis, we show different mobility shift patterns at -226 to -134 of the gamma-gene promoter in the hemin and HU treated cells. This suggests the possibility of a novel binding activity underlying the hemin and hydroxyurea effect.

The status of *in vivo* protein-DNA interactions in the promoter region of the G-gamma gene was investigated by a new ligation-mediated polymerase chain reaction (LMPCR), using N-ethyl,N-nitrosourea(ENU). We find that ENU acts efficiently on suspension cells and can detect protein-DNA interactions not revealed by the commonly used dimethyl sulfate(DMS) method. *In vivo* footprinting results suggest that both CCAAT sites are activated in gamma-globin gene transcription, octamer binding site is specific for hemin induction, and -50 and -200 regions have major roles in gamma-globin transcriptional. The protection pattern in the -200 region strongly suggests that repressor molecules bind in adult stage and activators in untreated and hemin treated K562 cells. Incubation in hydroxyurea inhibits binding at this region.

3) Pathophysiology of Sickle Cell Disease

Microvascular occlusion by poorly deformable erythrocytes is believed to be the key pathophysiologic event in sickle cell disease. A quantitative understanding of the molecular and cellular processes involved in the loss of deformability of sickle erythrocytes as compared to normal erythrocytes is crucial for clarification of the pathophysiology and treatment of this disease. The main determinant of Hb S polymer fraction in any population of sickle cells is oxygen saturation, and polymer fraction can be calculated as a function of oxygen saturation. However, the exact relationship between the deformability of intact erythrocyte and the formation of intracellular Hb S polymer is not yet well understood. On the other hand, both the abnormal membrane and the very high intracellular Hb concentrations in the populations of dense sickle cells also contribute to the rigidity of these cells. The relative contributions of these factors to cell rheology, as compared to polymerization, is also unknown. We, therefore, investigated the effects of these three factors: intracellular Hb S polymerization, the abnormal membrane, and the increased total intracellular Hb concentration in some cells on measured erythrocyte filterability (deformability) quantitatively, using a nickel mesh filtration system. In this study, we found by increasing the proportion of dense cells in populations of normal or sickle cells and equilibrating these cells with air or carbon monoxide (CO), that intracellular polymerization contributes about 4 times as much as intracellular viscosity and twice as much as abnormal membranes to impaired filterability, and that an increase in dense cells, which contained Hb S polymer due to the high cell hemoglobin concentration (CHC), impaired filtration significantly under air-equilibrated conditions. Moreover, we estimated that the filterability of erythrocytes containing Hb S was extremely sensitive to small amounts of intracellular polymer and that impaired filtration was almost linearly related to intracellular polymer fraction.

Variation in the amount of sickle hemoglobin produced in the erythrocyte is influenced by the production of other hemoglobins particularly in the case of sickle trait and other sickle cell syndromes. A detailed analysis in collaboration with investigators at Howard University School of Medicine of the solubility of mixtures of hemoglobin at various oxygen saturations provides the means to predict the maximum extent of polymerization within the sickle hemoglobin containing erythrocyte in various syndromes or in various individuals. A method to measure directly the equilibrium solubility of sickle hemoglobin mixtures with other hemoglobins at various oxygen saturations was developed. These measurements show that the solubility increases with increasing amounts of fetal hemoglobin at 0.32 g/dl per percent fetal hemoglobin. The solubility increases with ligand concentration as the critical oxygen saturation above which no polymer is detected is shifted to lower ligand concentrations as the percent fetal hemoglobin increases. These data are, we believe, the most accurate available as to the sparing effect of Hb F on polymerization.

Predictions of intracellular sickle hemoglobin polymerization tendency have been found to correlate strongly with severity of hemolysis in different sickle syndromes. Our analysis of 2674 individuals with sickle cell anemia from the Cooperative Study of Sickle Cell Disease database in conjunction with investigators from George Washington University indicate that polymerization tendency rises continuously during the first twelve years of life, due to continuous decrease in fetal hemoglobin and increase in mean corpuscular hemoglobin concentration until adolescence, and then becomes relatively constant. In general, males had higher polymerization tendencies than females, and individuals with alpha-thalassemia had lower values than those without alpha-thalassemia.

Polymerization tendency had a strong inverse correlation with hematocrit and correlated positively with frequency of pain crisis. These analyses may allow prediction of disease severity within cohorts of sickle cell anemia patients and provide a basis for evaluating therapeutic strategies designed to decrease intracellular sickle hemoglobin polymerization. A similar, but prospective "natural history" analysis has been initiated with several hospitals in Paris.

4) Human Erythropoietin Receptor

Erythropoietin (Epo) functions as a stimulating factor, resulting in proliferation of erythrocyte precursors and triggering differentiation to a mature erythrocyte or red blood cell. Therefore, it is likely that the cellular sensitivity to Epo greatly changes during the course of erythroid cell differentiation in parallel with the erythropoietin receptor (Epo-R) gene expression. We have recently cloned the genomic DNA of human Epo-R; in its 5' flanking region, there are potential regulatory sequences specific to the erythroid cell lineage. Therefore we speculate that 5' flanking region of the Epo-R gene is important for its regulation of expression at the transcriptional level. In order to examine the roles of these motifs on transcriptional regulation, we made plasmids containing deletions of the 5' flanking region with a reporter gene (luciferase) and transfected these constructs into cultured cells, OCIM-1 (high expressed Epo-R) and HeLa (no expressed Epo-R). Analysis of expression in OCIM-1 cells by luciferase assay indicates that -150 bp from the initiation start site is very critical for transcription. This region has an inverted GATA motif and short stretches of sequences which share homology with other transcriptional factors such as SP1. However, we did not detect any conspicuous events in other regions such as strong enhancers or silencers in the distal 5' region extending to about 2 kb 5' of the cap site. On the other hand, the result of experiments in HeLa cells demonstrated that the longest fragment (-937 to -1778) has transcriptional activity. These results demonstrate that non Epo-R expressing cell lines contain positive trans-acting factors that can activate the Epo-R enhancer and promoter in transient assays, yet probably the endogenous Epo-R in this cell is repressed. We suggest that repression of the chromosomal Epo-R gene in HeLa cells is a result of mechanisms that restrict accessibility of enhancer and promoter elements to trans-regulators possibly via changes in chromatin structure. In the erythroid system, Epo-R is likely regulated at multiple levels including chromatin structure, transcription regulation, and polypeptide translocation to the cell membrane resulting in modulation of Epo-R expression during erythroid maturation.

In gel mobility assays, nuclear extracts from erythroid and non-erythroid cell lines provide similar protein-DNA binding patterns to the proximal promoter. An additional band was observed in erythroid cell lines, likely corresponding to GATA-1 binding. In DNase I footprinting, we observed extensive protein binding to the region about -19, the location of the SP1 site, and the GATA-1 binding site at -47. Other protein binding regions are also detected within the proximal promoter including AP2 and another region with no known consensus sequences. Truncated deletion mutations of the regions extending beyond the cap site were linked to a luciferase reporter gene and assayed in OCIM1 erythroid cells by transient transfection. Deleting the unknown footprinted region at -151 in the promoter fragment resulted in an increase of activity to about 1.8 of the activity observed with the longer construct extending to 200 bp 5' indicating that the unknown footprint region may have a negative regulatory effect on the erythropoietin receptor promoter. Deleting the AP2 site reduced transcriptional activity to levels observed with the reference fragment indicating that the AP2 site may have an enhancing activity or a positive regulatory effect on the promoter activity. Surprisingly, deletion of the GATA-1 site at -47

in a promoter fragment reduced the transcriptional activity to only about 80% of the reference sequence. This suggests that while the GATA-1 site is able to enhance activity of the promoter, it is not absolutely required for promoter activity. This construct extending to only -29 leaves an SP1 site at -19 intact, suggesting that the SP1 site is sufficient to obtain at least minimal promoter activity. In contrast, deletion of these 5' sequences to +3 of the cap site markedly reduces luciferase activity to about 20% of the reference construct. These results suggest that SP1 is required for promoter activity and that GATA1 acts to further activate transcription.

We are presently studying the tissue and developmental specificity of the gene in transgenic mice. For these latter studies we have introduced the entire 15 kb cloned fragment of the hEpoR into mouse pronuclei by microinjection and generated several transgenic lines. Hematological parameters were measured in transgenic mice and found within normal limits except for a suggestion of increased reticulocyte levels. Tissue expression was determined in both normal and transgenic mice from several different lines. We found that mouse Epo-R transcripts were detected in bone marrow and spleen, but not in brain, heart, kidney and liver, but that human Epo-R transcripts were detected in the brain as well as in bone marrow and spleen, but not in heart, kidney and liver. No endogenous mEpoR were detected in brain of transgenic mice. Recently we have found that there is some expression of the endogenous gene very early in the brain but with development this decreases. We are currently studying the biological significance of these observations.

We previously reported the isolation and sequencing of the genomic and cDNA genes for the human erythropoietin receptor (hEpoR) and the development of a receptor transcript phenotype (RTP) assay based on the polymerase chain reaction (PCR). In this report we will discuss how these accomplishments have enabled us 1) to explore the role of the human EpoR in viability, proliferation, differentiation and signal transduction in erythropoiesis and 2) to begin to define sequences responsible for the appropriate regulation of the human EpoR. In the first project, both the normal and a mutationally activated cDNA gene for the human EpoR were cloned into expression vectors and electroporated into an interleukin-3 (IL-3) dependent murine myeloid progenitor cell line, 32D, which does not otherwise express the EpoR. Our analysis of these transformants indicate that both the wild type and mutant human EpoR can functionally substitute for the IL-3 receptor and support viability, proliferation and tyrosine phosphorylation in 32D cells. Transformants expressing the human EpoR do not exhibit overt erythroid differentiation nor are they inhibited from myeloid differentiation. In the second project we have begun to test if tissue and lineage specific transcription is appropriately regulated by sequences in the 5' flanking region of the human EpoR gene. We have made a mammalian expression vector which fuses the 5' flanking region of the human EpoR to the reporter gene beta-galactosidase and begun to study its regulation in different lineages or tissue culture cells.

We have used a recently developed primary erythroid culture system to study the effect of hydroxyurea in vitro. Using HPLC, we have been able to show an apparent increase in fetal hemoglobin per cell as well as in per cent fetal hemoglobin. Total hemoglobin per cell also appears to increase in thalassemic cultured cells. These results are similar to those found in vivo where treatment of sickle cell patients with hydroxyurea results in increases in percent fetal hemoglobin, fetal hemoglobin per cell and total hemoglobin per cell. We have been able to demonstrate that the effect of hydroxyurea on erythroid cells is occurring at a relatively late stage in erythroid maturation while the cytotoxicity of hydroxyurea toxicity is greatest at the earlier maturational stages, prior to the

onset of obvious hemoglobin formation. Also we have shown that hydroxyurea seems to increase the absolute amount of a modified hemoglobin. The significance of this finding is uncertain at this time. Increasing erythropoietin did not result in increased fetal hemoglobin. We believe that this non-neoplastic primary erythroid culture system will be a useful experimental system in which to examine in detail the molecular and cellular mechanism(s) of pharmacological induction of Hb F.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25008-28 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Core Loop Interaction That Controls Protein Folding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Hiroshi Taniuchi Chief, Section on Protein Chemistry LCB, NIDDK
and Conformation

Others: Alice Fisher Chemist LCB, NIDDK
Greg Charles Biological Aid LCB, NIDDK

COOPERATING UNITS (If any)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Protein Chemistry and Conformation

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

This project has been combined with project Z01 DK 25011-17 LCB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25011-18 LCB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Studies of Protein Folding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Hiroshi Taniuchi	Chief, Section on Protein Chemistry and Conformation	LCB, NIDDK
Others: Alice (Fisher) Hawley	Chemist	LCB, NIDDK
Greg Charles	Biological Aid	LCB, NIDDK
Yu-can Du	Guest Scientist	LCB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Protein Chemistry and Conformation

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.65	PROFESSIONAL: 1.2	OTHER: 0.45
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CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A long range goal of this project is to predict the three-dimensional structure of proteins on the basis of the amino acid sequence (a fundamental subject for structural biology). A ground work has been completed this year in that all previous studies of protein folding in this section are unified in a paper which is in press. Briefly, to study the mechanism of protein folding, we have developed the fragment complex system of cytochrome c. For example, Type I complex contains the discontinuity of the polypeptide chain between residues 24 and 25 and Type II between residues 38 and 39. Type IV lacks residues 26 to 38. Based on the studies of kinetics of folding and unfolding and the thermodynamic and structural properties of the complexes in the previous years, we have assigned four core domains. A core domain consists of a hydrophobic core and its surrounding shell and folds and unfolds as a unit. Core domain 1 contains most of the right channel structure, found by R. E. Dickerson and colleagues. It also includes a part of the heme. Core domains 2, 3 and 4, respectively are located on the left (the Fe-S bond) and right sides and the bottom (opposite to the right channel) of the heme. Type IV complex contains core domains 1 and 2. Core domain 2 of most of Type IV populations is unfolded at 25 degree C. Folding of this domain requires a decrease of the temperature or positioning of residues 28 to 38 (core domain 3). A combination of analysis of such core domain-domain interaction and kinetic studies in the previous years has led to a model of two alternative folding orders of the core domains of Type 1 complex: domain 1 to 3 to 2 to 4 or 1 to 2 to 3 to 4. Analysis of the data of standard Gibbs energy change for folding and heat stability of the Fe-S bond of various complexes containing substituted amino acids suggests that this folding is associated with an increase in the extent of the core group interaction. This interaction is apparently of long range and does not seem to involve solvent. We hypothesize that folding of Type I complex which resembles native cytochrome c is a process of unification of the ordered core of the core domains and that this unification is associated with expansion of the core group interaction to generate extra stabilizing energy. This hypothetical core group interaction is assumed to represent a new type of non-covalent interaction. Being consistent, the studies suggest that such core group interaction involving the side chain of leucine 32 strongly influences the stability of the Fe-S bond which is located at the core at a distance of 8 angstrom.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25016-19 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Trans-Acting Factor(s) Controlling Globin Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Alan N. Schechter

Chief

LCB, NIDDK

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Research Physicist

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Zi Yao Liu

Visiting Associate

LCB, NIDDK

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 0.3

PROFESSIONAL: 0.3

OTHER:

CHECK APPROPRIATE BOX(ES)



(a) Human subjects



(b) Human tissues



(c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have been studying the molecular genetic mechanisms that control the developmental switch from embryonic to fetal to adult hemoglobins with respect to their basic biology and their relevance to developing new therapies for diseases of hemoglobin. To study these processes we have been using two model systems: the K562 erythroleukemic cells and transgenic mice.

K562 is an erythroleukemic cell line used for the last decade as a model for the study of the control of human globin gene expression. These cells do not support transcription of the beta-globin gene but do express transcripts of epsilon- and gamma-globin genes at very high levels when exposed to a number of inducing agents. Results from this and other laboratories suggest that the control of this pattern of expression is mediated by the presence and/or absence of trans-acting factors which exert their action on sequences corresponding to the promoters of these genes. In the last few years transgenic mice have become an excellent model system for studying globin gene expression, and are supplementing the use of human cell lines.

We have previously reported the presence of a transcriptional control element with properties of a silencer extending from -392 to -177 bp relative to the cap site of the human epsilon-globin gene. Using deletion mutants and synthetic oligonucleotides in transient expression assays, DNA sequences responsible for this effect have been further delimited to 44 nucleotides located between -294 and -251 bp. Gel electrophoresis mobility shift assays and DNaseI footprinting assays demonstrate that these negative regulatory sequences are recognized differently by proteins present in nuclear extracts obtained from HeLa and K562 cells. The protein present in K562 cells, but not in HeLa cells, that interacts specifically with this silencer binds to the same sequence recognized by the yeast binding protein ABF1.

We have initiated studies using the transgenic technique to study the epsilon-globin silencer; constructs with the silencer sequence mutated are being compared to those with these nucleotides intact for their effect on expression of the epsilon globin gene in the embryonic and adult mouse. In addition, several lines of animals expressing the human alpha globin and beta-sickle-Antilles globin genes have been constructed and are being analyzed as possible models of the human disease, sickle cell anemia.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25021-17 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sickle Cell Anemia: The Intracellular Polymerization of HbS

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Constance Tom Noguchi Research Physicist LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK
Alan N. Schechter Chief LCB, NIDDK

COOPERATING UNITS (if any)

BEIB, NIH (R. Chadwick and C. Dong), George Washington University (L. Lessin), Howard University (W. Poillon), University of Mississippi School of Medicine, Jackson, MS (H. Steinberg), Nippon Medical School, Tokyo, Japan (N. Uyesaka)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, MD

TOTAL STAFF YEARS 0.3

PROFESSIONAL: 0.3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The reduced solubility of deoxygenated sickle hemoglobin leads to aggregation or polymerization of hemoglobin in sickle hemoglobin containing erythrocytes which can lead to abnormal rheology. Variation in the amount of sickle hemoglobin produced in the erythrocyte is influenced by the production of other hemoglobins particularly in the case of sickle trait and other sickle cell syndromes. The extent of polymerization of sickle hemoglobin is determined by oxygen saturation, hemoglobin concentration and hemoglobin composition. A detailed analysis of the solubility of mixtures of hemoglobin at various oxygen saturations provides the means to predict the maximum extent of polymerization within the sickle hemoglobin containing erythrocyte. A method to measure directly the equilibrium solubility of sickle hemoglobin mixtures with other hemoglobins at various oxygen saturations was developed. These measurements show that the solubility increases with increasing amounts of fetal hemoglobin at 0.32 g/dl per percent fetal hemoglobin. The solubility increases with ligand concentration as the critical oxygen saturation above which no polymer is detected is shifted to lower ligand concentrations as the percent fetal hemoglobin increases. A model has been developed using hydrodynamic lubrication theory to estimate the contributions of polymer and membrane changes to the impaired rheology of sickle erythrocytes. Predictions of intracellular sickle hemoglobin polymerization tendency have been found to correlate strongly with severity of hemolysis in different sickle syndromes. Analysis of 2674 individuals with sickle cell anemia from the Cooperative Study of Sickle Cell Disease database indicate that polymerization tendency rises continuously during the first twelve years of life, due to continuous decrease in fetal hemoglobin and increase in mean corpuscular hemoglobin concentration until adolescence, and then becomes relatively constant. In general, males had higher polymerization tendencies than females, and individuals with alpha-thalassemia had lower values than those without alpha-thalassemia. Polymerization tendency had a strong inverse correlation with hematocrit and correlated positively with frequency of pain crisis. These analyses may allow prediction of disease severity within cohorts of sickle cell anemia patients and provide a basis for evaluating therapeutic strategies designed to decrease intracellular sickle hemoglobin polymerization.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25025-16 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Mechanism of Antigen-Antibody Interaction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Hiroshi Taniuchi	Chief, Section on Protein Chemistry and Conformation	LCB, NIDDK
Others: Paola Rizzo	Visiting Fellow	LCB, NIDDK
Caterina Tinello	Visiting Fellow	LCB, NIDDK
Bridgette Person	Student Volunteer	LCB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry and Conformation

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 2.57

PROFESSIONAL: 2.4

OTHER: 0.17

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The X-ray structures of several Fab fragment-protein antigen complexes, determined by others, have revealed the details of the antigen-antibody interface. However, despite this, the antigen binding-interaction, which is fundamental for immunology, is not well understood. For example, a combination of hydrophobic interaction, van der Waals interaction, hydrogen bonds and electrostatic interaction is considered sufficient to describe the interaction. In the studies of protein folding (Project Z01 DK 25011-18 LCB) we have proposed the hypothesis that a long-range core group interaction exists to stabilize the structure. On the basis of this idea, to obtain a clue as to if such long-range core group interaction is also involved in the antigen-antibody interaction, we have studied amide hydrogen-exchange of three monoclonal IgG antibodies to yeast iso-1-cytochrome c and the Fab fragment of one of these antibodies in the presence and absence of the immunogen and evolutionarily related species. The study, which has been continuously carried out from the previous years, is now completed as a paper in press. Briefly, the number of hydrogens of the monoclonal antibodies whose exchange was suppressed on binding to the immunogen was found to exceed that estimated for the residues shielded by the immunogen. Analysis of the data suggests that such suppression of hydrogen exchange occurs mainly for the Fab domains but not for the Fc. One of the antibodies showed two distinct classes of amide-hydrogens. Class 1 hydrogens exchange faster than class 2. The exchange of class 1 hydrogens was suppressed by binding to the immunogen but not to the evolutionarily related species. The exchange of class 2 hydrogens was suppressed by binding to the evolutionarily related species as well as to the immunogen. Thus, the suppression of exchange of class 1 hydrogens appears to occur by some kind of conformational stabilization, the mechanism of which differentiates between the immunogen and the evolutionarily related species. Evidence suggests that the trans-interactions of the Fab domain may modulate the hydrogen exchange. If it is assumed that the antigen-binding strengthens the trans-interactions in such a way that the exchange of the slower exchanging hydrogens is suppressed, this could explain the suppression of exchange of class 2 hydrogens. The studies are consistent with the idea that the antigen-antibody interaction is not confined to the antigen-antibody interface and that it may involve some long-range interaction with distant residues of the Fab. As a step to achieve mapping of such residues, the variable domain of the heavy chain of one of the monoclonal antibodies has been essentially completely sequenced based on cDNA.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 25028-14 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Development of Non-Invasive Methods to Assess Sickle Cell Patients

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK

Others: Hiroyuki Hiruma Visiting Fellow LCB, NIDDK
Alan N. Schechter Chief LCB, NIDDK

COOPERATING UNITS (if any)

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BEIB (Eli Walker); Biometry Branch, NEI (M. Podgor); MRC Laboratory, Kingston, Jamaica
(G. Serjeant); Nippon Medical School (Dr. N. Uyesaka)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Unit on Molecular Hematology

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been temporarily suspended; it will be resumed in the next reporting period.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25045-08 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Beta Globin Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. (Name, title, laboratory, and institute affiliation))

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

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- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

Project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25058-07 LCB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Laboratory Model of Adult Globin Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
PI: David Ebb Special Volunteer LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK
In-Hoo Kim Visiting Fellow LCB, NIDDK
Alan N. Schechter Chief LCB, NIDDK

COOPERATING UNITS (if any)
Children's Hospital National Medical Center, Washington, D.C. (Dr. D. Ebb)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.0 PROFESSIONAL: 1.0 OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are investigating the molecular mechanisms which control the individual and total concentrations of hemoglobins in human erythrocytes. The study of the control of globin gene expression has direct relevance to the development of therapies for various hemoglobinopathies. We are particularly interested in sickle cell anemia and beta-thalassemia, where mutations or deletions of adult globin genes can produce clinical syndromes of severe anemia and progressive organ damage.

Our current experimental system uses a tissue culture model of gene expression, with future plans to study globin gene regulation in transgenic mice. Present efforts are focused on elucidating the mechanisms controlling globin gene transcription in the K562 erythroleukemia cell line. Previous studies of K562 cells grown in the presence of hemin or 5-azacytidine have demonstrated inducible expression of globin genes with an embryonic-fetal phenotype. S1 nuclease mapping of globin gene transcripts has revealed constitutive low level, expression of the adult delta globin gene, which has proven to be inducible with hemin. Adult beta globin gene transcripts, however, are undetectable both in uninduced and induced K562 cells. This phenotype contrasts markedly with normal adult erythrocytes where beta-globin is expressed at levels 40 times higher than delta globin.

Comparisons of beta and delta globin sequence information has shown near identity of sequence in the transcribed region. In the 5' flanking region, however, the delta and beta globin genes share only 30% homology. We are exploring the possibility that discrepancies in the expression levels of these two adult genes may be due to differential binding of trans-acting proteins to upstream regulatory regions. This investigation has so far entailed the cloning of the promoter regions of the epsilon, delta and beta globin genes into the eukaryotic expression vector PSVOCAT. We have also cloned additional CAT vector constructs which incorporate chimeric promoters consisting of both delta and beta globin promoter elements. These constructs will be transfected into K562 cells to study the interaction of the trans-acting factors unique to this cell line, with the cis-elements contained in the globin gene promoter regions. In conjunction with functional assays of relative promoter strength, we will perform in vitro footprinting on these same promoter elements to define binding sites of putative trans-acting regulatory proteins. Identification of these proteins may not only provide important information on the regulation of minor hemoglobin synthesis, but may facilitate identification of the trans-acting factors responsible for beta globin gene expression.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25059-06 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Trans-activating Factors and Globin Gene Expression: A Direct Approach

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Harish Dave

Visiting Fellow

LCB, NIDDK

Others: Alan N. Schechter

Chief

LCB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS.

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
☐ (a1) Minors
☐ (a2) Interviews

☒ (b) Human tissues

☐ (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been combined with Project Z01 DK 25016-18 LCB.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25060-07 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Epsilon-Globin Gene Silencer: Characterization of a Trans-acting Factor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Constance Tom Noguchi Research Physicist LCB, NIDDK

Others: Betty Peters

Guest Worker

LCB, NIDDK

COOPERATING UNITS (if any)

Nippon Medical School, Tokyo, Japan (Y. Wada-Kiyama)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.2

PROFESSIONAL: 1.2

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (a1) Minors☐ (a2) Interviews☐ (b) Human tissues☒ (c) Neither

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

K562 human erythroleukemia cells constitutively express epsilon- and gamma- but not beta-globin genes. We have previously shown that the differential expression of globin genes observed in intact K562 cells could be simulated in vitro as K562 nuclear extract (NE) actively transcribes the epsilon-globin (with 2 kb of 5' flanking sequence) and gamma-globin gene DNA templates but not beta-globin gene templates. We have now used the K562 in vitro transcription system to examine a silencer transcriptional control element which has been reported to be localized between -177 and -392 bp 5' of the canonical cap site for the epsilon-globin gene. We find that K562 NE has markedly reduced synthesis of RNA in vitro from epsilon-globin gene DNA deletion templates which contain the silencer sequence, or part thereof, but not the adjacent 5' positive regulatory region (-453 to -535 bp). Furthermore, those transcripts generated in vitro from DNA templates extending to -453 bp or less of the epsilon-globin gene were not correctly initiated at the canonical cap site. Separating the K562 NE by ion exchange chromatography, we isolated a fraction (F175) transcriptionally active for all tested globin genes including the epsilon-globin gene containing the silencer sequence and a fraction (F50) which contains the trans-acting factors associated with the silencer activity. F50 showed a strong dose dependent inhibitory effect on correctly initiated epsilon-globin gene transcription directed by either unfractionated K562 NE or F175. This suppression by F50 was not observed on transcriptional activity of the permissive adenovirus 2 major late promoter. In electrophoretic mobility shift assays using the epsilon-globin gene silencer region as probe, F50 and F175 exhibited different DNA binding protein patterns: a specific protein band in F50 appears to be associated with the silencer activity. These studies suggest that this protein may be specifically responsible for the activity of the silencer element of the epsilon-globin gene. The expression and silencing of the epsilon-globin gene during development may be modulated by the interactions of this protein with the cis-acting DNA silencer.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 25061-07 LCB									
PERIOD COVERED October 1, 1991 to September 30, 1992											
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Expression of Human Erythropoietin Receptor Gene in Transgenic Mice											
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Z. Y. Liu</td> <td style="width: 33%; text-align: center;">Visiting Associate</td> <td style="width: 33%; text-align: right;">LCB, NIDDK</td> </tr> <tr> <td>Others: Alan N. Schechter</td> <td style="text-align: center;">Chief</td> <td style="text-align: right;">LCB, NIDDK</td> </tr> <tr> <td style="padding-left: 40px;">Constance T. Noguchi</td> <td style="text-align: center;">Research Physicist</td> <td style="text-align: right;">LCB, NIDDK</td> </tr> </table>			PI: Z. Y. Liu	Visiting Associate	LCB, NIDDK	Others: Alan N. Schechter	Chief	LCB, NIDDK	Constance T. Noguchi	Research Physicist	LCB, NIDDK
PI: Z. Y. Liu	Visiting Associate	LCB, NIDDK									
Others: Alan N. Schechter	Chief	LCB, NIDDK									
Constance T. Noguchi	Research Physicist	LCB, NIDDK									
COOPERATING UNITS (if any)											
LAB/BRANCH Laboratory of Chemical Biology											
SECTION Section on Molecular Biology and Genetics											
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland											
TOTAL STAFF YEARS 1.1	PROFESSIONAL: 1.1	OTHER:									
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input type="checkbox"/> (b) Human tissues</td> <td><input checked="" type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews		
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither									
<input type="checkbox"/> (a1) Minors											
<input type="checkbox"/> (a2) Interviews											
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Erythropoietin (Epo) is a primary regulator of erythropoiesis and functions by binding to the erythropoietin receptor (EpoR) on the surface of hematopoietic progenitor cells, followed by receptor-mediated endocytosis of the Epo molecule. The human EpoR (hEpoR) has been known to consist of two molecular weight polypeptides (85 kd and 100 kd) and to exhibit both low and high affinity binding sites for Epo. We have recently cloned and sequenced the gene for the main polypeptide of the hEpoR (Noguchi, et al <i>BLOOD</i>, 78:2548-2556, 1991). The structure of the gene with respect to introns and exons and possibly transcription regulatory sequences in the DNA surrounding the gene has been elucidated. We are presently studying the tissue and developmental specificity of the gene in transient assays (see Project Number Z01 25082-012 LCB) and, as described in this report, in transgenic mice. For these latter studies we have introduced the entire 15 kb cloned fragment of the hEpoR into mouse pronuclei by microinjection and generated several transgenic lines. Hematological parameters were measured in transgenic mice and found within normal limits except for a suggestion of increased reticulocyte levels. Tissue expression was determined in both normal and transgenic mice from several different lines. We found that mouse Epo-R transcripts were detected in bone marrow and spleen, but not in brain, heart, kidney and liver, but that human Epo-R transcripts were detected in the brain as well as in bone marrow and spleen, but not in heart, kidney and liver. No endogenous mEpoR were detected in brain of transgenic mice. Recently we have found that there is some expression of the endogenous gene very early in the brain but with development this decreases. We are currently studying the biological significance of these observations. </p>											

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25063-06 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of Hydroxyurea on Fetal Hemoglobin Synthesis in Congenital Hemoglobin Disorders

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK

Others: Shu-zhen Huang	Visiting Associate	LCB, NIDDK
Hiroyuki Hiruma	Visiting Fellow	LCB, NIDDK
Constance T. Noguchi	Research Physicist	LCB, NIDDK
Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

CHB, NHLBI (A.W. Nienhuis); Depts. of Medicine, Pediatrics & Pathology, Johns Hopkins University, Baltimore, MD (Dr. G. Dover); Laboratory of Medical Genetics, Shanghai's Children's Hospital, Shanghai, China (Drs. Y. Zeng and S. Huang)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Unit on Molecular Hematology

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 0.9

PROFESSIONAL: 0.9

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our group has focused on pharmacologic manipulation of fetal hemoglobin levels in patients with genetic defects of hemoglobin. We have previously shown that hydroxyurea (HU) treatment of severely affected sickle cell patients results in about a 70 to 75% response rate defined by at least a two-fold increase in F-reticulocytes and a concomitant two-fold rise in per cent in hemoglobin F. The best responding patients achieved levels of fetal hemoglobin of 10 to 15%. We have subsequently treated 6 patients with beta thalassemia intermedia with HU. Two patients showed an increase of at least 2-fold in HbF levels and of 5-fold in gamma globin mRNA. Curiously, two other patients showed an obvious increase primarily in beta-globin biosynthesis corresponding to an increase in beta-mRNA transcripts without a change in gamma globin synthesis. Thus, in addition to its effects in stimulating gamma globin synthesis, HU may be useful in the context of treatment of beta thalassemia through other mechanisms. In an effort to achieve higher levels of hemoglobin F, in a more pancellular distribution, we have recently treated four patients with sickle cell disease who were receiving hydroxyurea for periods of 5-15 months on four consecutive days with escalating dose of recombinant erythropoietin (EPO) for 7 weeks, given on the alternate three days along with oral iron sulfate. Treatment with EPO in combination with chronic hydroxyurea therapy had a significant effect on the percentage of hemoglobin F containing reticulocytes (F-reticulocytes), and red cells (F-cells) and on the total hemoglobin F level. In these four patients such combination therapy was associated with a 1.4 to 3 fold increase in F-reticulocytes, a 1.3 to 2 fold increase in F-cells and a 1.4 to 2 fold increase in the percentage of hemoglobin F when compared to the maximal values previously achieved on hydroxyurea alone. In addition, in contrast to the results on hydroxyurea alone, treatment with combination HU/EPO resulted in maximal stimulatory effects within ten to twelve days of therapy. This additional augmentation resulted in a further decrease in serum bilirubin and lactate dehydrogenase as well as a further decline in intracellular hemoglobin S polymerization tendency at physiologic oxygen saturation in cells containing fetal hemoglobin. We conclude that (1) while HU is a potent inducer of gamma-globin synthesis, it may have a more generic effect on globin gene expression; (2) when given in an alternating fashion with erythropoietin in addition to iron supplementation it tends to augment the fetal hemoglobin responses seen with hydroxyurea alone.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25064-06 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cytogenetic Investigations of Patients With Genetically Determined Disorders

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Beverly J. White Director, Cytogenetic Unit LCB, NIDDK

Others: Damrong Wangsa Photographer, Scientific Technical OD, CC
Flanagan Whitsitt Medical Technician OD, CC
Cynthia Powell Clinical Associate OD, CC
Vincent Obias Student Volunteer OD, CC

COOPERATING UNITS (if any)

Medical Genetics Program, CC (W. Gahl, D. Parry); DE, NICHD (G. Cutler, L. Nelson); LN, NIA (M. Schapiro, S. Rapoport); Dept. Molecular Biology & Genetics, Wayne State University School of Medicine, Detroit (R.T. Taggart).

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Molecular Forces and Assembly (Cytogenetics Unit)

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 4

PROFESSIONAL: 3.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cytogenetic analyses of 221 patients in clinical center protocols were reported. Clinical studies of several endocrine disorders required confirmation of cytogenetic diagnosis and detection of genetic variation possibly affecting therapeutic response. Cytogenetic screening was also done to determine protocol eligibility or to reveal constitutional aberrations which might reveal gene localization in unmapped single gene disorders. In some diseases, we searched for secondary aberrations which might play a role in development of clinical abnormalities.

No cytogenetic abnormalities were seen in 29 new patients with mental disorders. However, a translocation previously detected in a schizophrenic patient was found in his relatives; cell lines were established from the family, but linkage studies were deferred because the rearrangement and mental problems were not consistently associated.

Fluorescent in-situ hybridization (FISH) with chromosome-specific satellite DNA probes was used for investigation of a patient with premature ovarian failure and an X-autosome rearrangement, twin sisters with the Turner syndrome and a minute Y chromosome, a previously reported patient with mosaic Down syndrome, and a 46,XX male with hermaphroditism. Cell lines from several of these patients are being studied to detect and localize molecular defects and correlate them with clinical manifestations.

The unit is affiliated with the NIH medical genetics program, is accredited by the ABMG to provide post-doctoral training in clinical laboratory cytogenetics, and provides clinical consultations. It also participates in the College of American Pathologists cytogenetics proficiency testing program, which is required for accreditation of the genetics and pathology training programs.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25066-06 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

AIDS: Transcriptional Regulation by TAT-Protein and LTR of HIV In Vitro

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Jiangang Yuan Visiting Associate LCB, NIDDK

Others: Tzee-chen Shieh Visiting Fellow LCB, NIDDK
Constance T. Noguchi Research Physicist LCB, NIDDK
Alan N. Schechter Chief LCB, NIDDK

COOPERATING UNITS (if any)

Kabigen, Stockholm, Sweden (Professor M. Hartmanis)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been discontinued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25069-03 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Coordinated Expression of Human Beta Sickle Antilles and Human Alpha Globin in Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Frank Shafer	NRSA Fellow	LCB, NIDDK
Others: Zi Yao Liu	Visiting Associate	LCB, NIDDK
Constance T. Noguchi	Research Physicist	LCB, NIDDK
Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

DMBN, NIND (Drs. S. Karlsson and B. Dropulic); University of South Carolina, Columbia, S.C. (Dr. M. Dewey); Nippon University, Tokyo (Dr. N. Uyesaka)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER 0

CHECK APPROPRIATE BOX(ES)

- | | | |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors | | |
| <input type="checkbox"/> (a2) Interviews | | |

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

This project has been combined with Project Z01 DK 25016-18 LCB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25070-04 LCB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Analysis of the Epsilon Globin Gene Flanking Sequences

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
PI: Panagoula Kollia Visiting Fellow LCB, NIDDK

Others: Kyung Chin Research Biologist LCB, NIDDK
Constance T. Noguchi Research Physicist LCB, NIDDK
Alan N. Schechter Chief LCB NIDDK

COOPERATING UNITS (if any)
Hahneman Medical School (Dr. Doris Morgan), Philadelphia, Pennsylvania

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.3 PROFESSIONAL: 1.3 OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The developmental switch in hemoglobin synthesis, from embryonic (including epsilon globin) to fetal (gamma) to adult (beta) hemoglobins, that occurs during ontogeny is of great interest because of its fundamental biology as well as its relevance to the treatment of hemoglobinopathies. We have been studying transcriptional regulation of globin genes to clarify the molecular mechanisms of this switch. Within the cell, regulation of transcriptional activity of globin genes is determined by a variety of cis-acting regulatory DNA sequences and trans-acting proteins. One of the cis-acting elements is the silencer located in the region between -177 bp and -392 bp 5' to the epsilon globin gene, which was characterized in this laboratory.

To delineate the molecular mechanism of the epsilon globin gene silencer activity and its possible role in the developmental regulation of epsilon gene expression, further characterization of epsilon gene transcription initiation sites in cell systems where the epsilon gene is up and down regulated, such as MB-02 and the two-phase liquid culture system of Fibach, was studied. A variety of clues in the literature and in our own data suggested that the epsilon silencer could affect transcription initiation. The K562 erythroleukemia cell line constitutively expresses low levels of embryonic and fetal, but not adult hemoglobin and can serve as a control. We have now shown that in NB-02 cells levels of correctly initiated epsilon transcripts are significantly lower than in K562 cells. Correctly initiated epsilon transcripts were not detectable in normal cells prepared in the liquid culture system. We have, however, observed a smaller band in T1 analysis using a specific probe for exon I and 5' upstream region of the epsilon globin gene. Moreover, using the exon II of the epsilon gene as probe in T1 analysis we have detected the expected band and also a few smaller bands. Quantitating of epsilon exon I transcripts using cDNA amplification showed a small amount of correctly initiated epsilon transcripts in normal cells. Further characterization of the smaller bands will test the possible role of the epsilon gene silencer in regulation of developmental expression of epsilon gene. The data so far are compatible with our original hypothesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25071-04 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Trans-Regulation of Human Globin Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Robert H. Broyles

Guest Worker

LCB, NIDDK

Others: Patricia Berg

Expert

LCB, NIDDK

Alan N. Schechter

Chief

LCB, NIDDK

COOPERATING UNITS (if any)

Department of Biochemistry, University of Oklahoma School of Medicine (Dr. R. Broyles)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

NIDDK, Bethesda, Maryland

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been discontinued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25073-04 LCB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
The Erythropoietin Receptor and its Genetic Control in Red Cell Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Donna Williams Special Volunteer LCB, NIDDK

Others: Teresa A. Zimmers Special Volunteer LCB, NIDDK
Eitan Fibach Visiting Associate LCB, NIDDK
Alan N. Schechter Chief LCB, NIDDK

COOPERATING UNITS (if any)

Johns Hopkins University, Baltimore, MD (Drs. W. David Hankins and Donna Williams); LCMB, NCI (Dr. J. H. Pierce)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.2 PROFESSIONAL: 1.2 OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We previously reported the isolation and sequencing of the genomic and cDNA genes for the human erythropoietin receptor (hEpoR) and the development of a receptor transcript phenotype (RTP) assay based on the polymerase chain reaction (PCR). In this report we will discuss how these accomplishments have enabled us 1) to explore the role of the human EpoR in viability, proliferation, differentiation and signal transduction in erythropoiesis and 2) to begin to define sequences responsible for the appropriate regulation of the human EpoR. In the first project, both the normal and a mutationally activated cDNA gene for the human EpoR were cloned into expression vectors and electroporated into an interleukin-3 (IL-3) dependent murine myeloid progenitor cell line, 32D, which does not otherwise express the EpoR. Our analysis of these transformants indicate that both the wild type and mutant human EpoR can functionally substitute for the IL-3 receptor and support viability, proliferation and tyrosine phosphorylation in 32D cells. Transformants expressing the human EpoR do not exhibit overt erythroid differentiation nor are they inhibited from myeloid differentiation. In the second project we have begun to test if tissue and lineage specific transcription is appropriately regulated by sequences in the 5' flanking region of the human EpoR gene. We have made a mammalian expression vector which fuses the 5' flanking region of the human EpoR to the reporter gene beta-galactosidase and begun to study its regulation in different lineages of tissue culture cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 25074-04 LCB
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT <i>(80 characters or less. Title must fit on one line between the borders.)</i> Mechanism(s) of Enhanced Gamma Globin Gene Expression in Patients		
PRINCIPAL INVESTIGATOR <i>(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)</i> PI: In-Hoo Kim Visiting Fellow LCB, NIDDK		
Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK Alan N. Schechter Chief LCB, NIDDK Eitan Fibach Visiting Associate LCB, NIDDK		
COOPERATING UNITS <i>(if any)</i> University of Maryland School of Medicine, Baltimore, Maryland (P. Berg)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL STAFF YEARS 1.2	PROFESSIONAL: 1.2	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK <i>(Use standard unredacted type. Do not exceed the space provided.)</i> Several lines of clinical and experimental evidence suggest that elevated levels of fetal hemoglobin (HbF) may improve the clinical course of individuals with sickle cell disease and beta-thalassemia. A number of cytotoxic drugs have been shown to enhance gamma-globin synthesis (and HbF levels) in animals and patients with hemoglobinopathies, although the mechanism of action of these is not known. The K562 human erythroleukemia cell line shows constitutively low levels of embryonic and fetal hemoglobin, and can be reversibly induced to increase gamma-globin gene expression in response to hydroxyurea and other agents. We are therefore using the K562 cell as a model system to understand the mechanism of induction of globin gene transcription. K 562 cells have been grown in the presence of 25mM hemin and 100mM hydroxyurea. We have used gel-retardation electrophoresis, DNA-footprinting (DNase I and methylation protection), in vivo genomic DNA footprinting, and ion exchange and affinity chromatography to investigate the interaction of both specific and non-specific proteins to globin gene promoter DNA. By gel shift analysis, we show different mobility shift patterns at -226 to -134 of the gamma-gene promoter in the hemin and HU treated cells. This suggests the possibility of a novel binding activity underlying the hemin and hydroxyurea effect. We will localize and specify this binding site by competition assay with specific probes, DNaseI footprinting, and in vivo genomic DNA footprinting analysis. The status of in vivo protein-DNA interactions in the promoter region of the G-gamma gene was investigated by a new ligation-mediated polymerase chain reaction (LMPCR), using N-ethyl,N-nitrosourea(ENU). We find that ENU acts efficiently on suspension cells and can detect protein-DNA interactions not revealed by the commonly used dimethyl sulfate(DMS) method. In vivo footprinting results suggest that both CCAAT sites are activated in gamma-globin gene transcription, octamer binding site is specific for hemin induction, and -50 and -200 regions have major roles in gamma-globin transcriptional. The protection pattern in the -200 region strongly suggests that repressor molecules bind in adult stage and activators in untreated and hemin treated K562 cells. Incubation in hydroxyurea inhibits binding at this region. It is hoped that the further identification, characterization and purification of these putative binding proteins would not only extend the current knowledge of the molecular basis of the fetal to adult "switch", but also suggest novel pharmacological approach to the reversal of this switch in several clinically significant hemoglobinopathies.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25075-03 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992.

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Production and Characterization of HIV TAT

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Tice-Cheng Shieh	Visiting Fellow	LCB, NIDDK
Others: Jian-gang Yuan	Visiting Fellow	LCB, NIDDK
Constance T. Noguchi	Research Physicist	LCB, NIDDK
Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

Kabigen AB, Stockholm, Sweden (Dr M. Hartmanis), LCB, NIDDK, (Dr. A. Gronenborn), University of Padova, Italy (Dr. C. DiBello)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER: 0

CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been combined with Project Z01 DK 25066-05 LCB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 25076-03 LCB
PERIOD COVERED October 1, 1991 to September 31, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Characterization of the Epsilon-Globin Silencer		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> PI: Betty Peters Robert Wood Johnson Fellow LCB, NIDDK </div>		
Others: Constance Tom Noguchi Research Physicist LCB, NIDDK		
COOPERATING UNITS (If any)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL STAFF YEARS 1.2	PROFESSIONAL: 1.2	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div> <input type="checkbox"/> (b) Human tissues </div> <div> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Hemoglobin switching is an example of tissue specific and temporal regulation of gene expression. In humans, embryonic globin chains (zeta and epsilon) are expressed during the first trimester of gestation followed by a switch to the expression of fetal globin chains early in the second trimester and adult globin chains after birth. Previous work performed in the Laboratory of Chemical Biology has identified a silencer 200 to 400 bp upstream of the epsilon-globin gene which has been implicated in suppressing epsilon globin gene expression during the fetal and adult stages. Our work has focused on characterizing proteins binding to the silencer and the function of the binding of trans-acting factors to the silencer. We are in the process of elucidating the mechanism(s) that trans-acting factors interacting with the silencer utilize in order to regulate epsilon-globin transcription. We are also studying the interactions of the silencer with other regulatory elements controlling epsilon-globin gene expression. Using DNase I footprinting, we have identified several sites of protein binding to the silencer. The major protected region shares a high percentage of homology with the binding sites of an erythroid specific transcription factor GATA-1 and a yeast silencer binding protein, ABF-1. Using gel mobility shift assays with K562 nuclear extract, yeast ABF-1 protein and a probe bracketing the major protected region and competitor DNA with mutations in the sites homologous to GATA-1 and ABF-1 binding sites we have demonstrated that a protein, likely GATA-1, binds to the GATA site in the silencer and that another protein, likely a human homologue of the yeast ABF-1 protein, binds to the silencer. We are currently in the process of examining the function of mutations made in the yeast ABF-1 and GATA binding sites. We are also in the process of cloning the human homolog of the yeast ABF-1 gene. </p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25077-03 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Globin Gene Expression and the Treatment of Hemoglobinopathies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Shu-zhen Huang Visiting Associate LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology LCB, NIDDK

Alan N. Schechter Chief LCB, NIDDK

Mai-Jue Chen Special Volunteer LCB, NIDDK

COOPERATING UNITS (if any)

Institute of Medical Genetics and Children's Hospital, Shanghai, China (Drs. Y.-T. Zeng, Zhao-rui Ren, Ying Huang, Zhi-hong Lu and Jiang Hui)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS

1.4

PROFESSIONAL:

1.4

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The beta-thalassemias and sickle cell disease represent the most common congenital red cell disorders resulting from genetic defects in beta-globin gene expression. Recently, hydroxyurea (HU) has been shown to increase fetal hemoglobin (HbF) production in sickle cell patients and is also under evaluation as a treatment for beta-thalassemia. However, the effects of HU treatment on globin gene expression, particularly at the mRNA level, is not well understood. In order to improve our understanding of the mechanisms of action of HU, two HbSS patient and two beta-thalassemia patient (with IVS-2 nt 654 cytosine to thymine, CD 41-42 mutations) who received HU treatment were studied for changes in alpha, beta, and gamma mRNA levels by our newly developed competitive polymerase chain reaction (PCR) method. PCR quantitation was achieved by reverse transcriptase (RT) production of cDNA from mRNA in nucleated peripheral blood cells and subsequent competitive analysis of absolute mRNA levels. The results showed that the gamma-globin mRNA amount was increased by about 50-fold and the absolute magnitude of the change in gamma-mRNA levels were similar in the SS and beta-thalassemia patients. Increases, but to a smaller extent, were also observed in alpha and beta globin mRNA levels. These results suggest that the molecular mechanism of HU (and perhaps other related compounds) is a general one on expression of all globin genes, but greatest for gamma. Current studies in progress are aimed at: (1) investigation of the correlations between the globin mRNA levels and the reticulocyte numbers in nucleated cells when using the competitive RT-PCR method for globin mRNA assay; (2) investigation of globin gene expression (mRNA levels) by RT-PCR method in erythroid cultures treated with hydroxyurea; (3) investigation of globin gene expression (mRNA levels) in HU treated erythroid cultures from the patients with different types of beta-thalassemia mutations; (4) globin chain assay in HU treated patients as well as erythroid cultures by micro-globin biosynthesis assay. Thus, the overall objective is to contribute to the knowledge of the effects of HU on genetic regulation of globin production, and to provide us with understanding of the gamma to beta or beta to gamma switches in the synthesis of hemoglobin and how these switches may be modulated by drugs such as HU and erythropoietin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 25078-02 LCB
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Globin Expression in an Erythroid Progenitor Culture System		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> PI: Lillian Burke </div> <div style="width: 30%;"> NRSA Fellow </div> <div style="width: 30%;"> LCB, NIDDK </div> </div> <div style="margin-top: 10px;"> Others: Eitan Fibach Visiting Associate LCB, NIDDK Constance T. Noguchi Research Physicist LCB, NIDDK Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK Alan N. Schechter Chief LCB, NIDDK </div>		
COOPERATING UNITS (if any) Children's Hospital Research Institute (E. Witkowski), Oakland, CA		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, MD		
TOTAL STAFF YEARS 1.4	PROFESSIONAL: 1.4	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (b) Human tissues </div> <div style="width: 30%;"> <input type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>During normal development, a switch from the production of fetal hemoglobin (containing gamma chains) to the production of adult hemoglobin (containing beta chains) occurs. The gamma to beta switch in the synthesis of hemoglobins is not well understood. Hydroxyurea is used in the treatment of sickle cell anemia to increase the relative amount of fetal to sickle hemoglobin. Studies of hemoglobin switching and of drugs which affect it, such as hydroxyurea, have been difficult due to the absence of a culture system in which erythroid progenitors could be isolated in sufficient numbers to perform molecular studies.</p> <p>We have used a recently developed primary erythroid culture system (Fibach, 1989) to study the effect of hydroxyurea in vitro. Using HPLC, we have been able to show an apparent increase in fetal hemoglobin per cell as well as in per cent fetal hemoglobin. Total hemoglobin per cell also appears to increase in thalassemic cultured cells. These results are similar to those found in vivo where treatment of sickle cell patients with hydroxyurea results in increases in percent fetal hemoglobin, fetal hemoglobin per cell and total hemoglobin per cell.</p> <p>We have been able to demonstrate that the effect of hydroxyurea on erythroid cells is occurring at a relatively late stage in erythroid maturation while the cytotoxicity of hydroxyurea toxicity is greatest at the earlier maturational stages, prior to the onset of obvious hemoglobin formation. Also we have shown that hydroxyurea seems to increase the absolute amount of a modified hemoglobin. The significance of this finding is uncertain at this time. Increasing erythropoietin did not result in increased fetal hemoglobin.</p> <p>We believe that this non-neoplastic primary erythroid culture system will be a useful experimental system in which to examine in detail the molecular and cellular mechanism(s) of pharmacological induction of Hb F.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25079-01 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Development of Rheological Methods to Assess Sickle Cell Patients

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Hiroyuki Hiruma Visiting Fellow LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK
Alan N. Schechter Chief LCB, NIDDK
Constance T. Noguchi Research Physicist LCB, NIDDK

COOPERATING UNITS (if any)

Nippon Medical School, Tokyo, Japan (N. Uyesaka)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.3

PROFESSIONAL: 1.3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Microvascular occlusion by poorly deformable erythrocytes is believed to be the key pathophysiological event in sickle cell disease. A quantitative understanding of the molecular and cellular processes involved in the loss of deformability of sickle erythrocytes as compared to normal erythrocytes is crucial for clarification of the pathophysiology and treatment of this disease. Polymerization of hemoglobin S (Hb S) within sickle erythrocytes is presumably one of the most important factors in the loss of deformability. The main determinant of Hb S polymer fraction in any population of sickle cells is oxygen saturation, and polymer fraction can be calculated as a function of oxygen saturation. However, the exact relationship between the deformability of intact erythrocyte and the formation of intracellular Hb S polymer is not yet well understood. On the other hand, both the abnormal membrane and the very high intracellular Hb concentrations in the populations of dense sickle cells also contribute to the rigidity of these cells. The relative contributions of these factors to cell rheology, as compared to polymerization, is also unknown. We, therefore, investigated the effects of these three factors: intracellular Hb S polymerization, the abnormal membrane, and the increased total intracellular Hb concentration in some cells on measured erythrocyte filterability (deformability) quantitatively, using a nickel mesh filtration system. In this study, we found by increasing the proportion of dense cells in populations of normal or sickle cells and equilibrating these cells with air or carbon monoxide (CO), that intracellular polymerization contributes about 4 times as much as intracellular viscosity and twice as much as abnormal membranes to impaired filterability, and that an increase in dense cells, which contained Hb S polymer due to the high cell hemoglobin concentration (CHC), impaired filtration significantly under air-equilibrated conditions. Moreover, we estimated that the filterability of erythrocytes containing Hb S was extremely sensitive to small amounts of intracellular polymer and that impaired filtration was almost linearly related to intracellular polymer fraction. Thus, the polymerization of Hb S would be expected to be a much more important factor in loss of filtration in microcirculation where oxygen tension is low. These results emphasize the importance of Hb S polymerization in the pathogenesis of sickle cell disease, and will help define the pathophysiology of sickle cell disease in quantitative terms.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25080-01 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transcriptional Regulation of Human Erythropoietin-receptor Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Naoka Oda

Visiting Fellow

LCB, NIDDK

Others: Constance Tom Noguchi

Research Physicist

LCB, NIDDK

COOPERATING UNITS (If any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.1

PROFESSIONAL: 1.1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Erythropoietin (Epo) functions as a stimulating factor, resulting in proliferation of erythrocyte precursors and triggering differentiation to a mature erythrocyte or red blood cell. Therefore, it is likely that the cellular sensitivity to Epo greatly changes during the course of erythroid cell differentiation in parallel with the erythropoietin receptor (Epo-R) gene expression. We have recently cloned the genomic DNA of human Epo-R; in its 5' flanking region, there are potential regulatory sequences specific to the erythroid cell lineage. Therefore we speculate that 5' flanking region of the Epo-R gene is important for its regulation of expression at the transcriptional level. In order to examine the roles of these motifs on transcriptional regulation, we made plasmids containing deletions of the 5' flanking region with a reporter gene (luciferase) and transfected these constructs into cultured cells, OCIM-1 (high expressed Epo-R) and HeLa (no expressed Epo-R).

Analysis of expression in OCIM-1 cells by luciferase assay indicates that -150 bp from the initiation start site is very critical for transcription. This region has an inverted GATA motif and short stretches of sequences which share homology with other transcriptional factors such as SP1. However, we did not detect any conspicuous events in other regions such as strong enhancers or silencers in the distal 5' region extending to about 2 kb 5' of the cap site. On the other hand, the result of experiments in HeLa cells demonstrated that the longest fragment (-937 to -1778) has transcriptional activity. These results demonstrate that non Epo-R expressing cell lines contain positive trans-acting factors that can activate the Epo-R enhancer and promoter in transient assays, yet probably the endogenous Epo-R in this cell is repressed. We suggest that repression of the chromosomal Epo-R gene in HeLa cells is a result of mechanisms that restrict accessibility of enhancer and promoter elements to trans-regulators possibly via changes in chromatin structure. In the erythroid system, Epo-R is likely regulated at multiple levels including chromatin structure, transcription regulation, and polypeptide translocation to the cell membrane resulting in modulation of Epo-R expression during erythroid maturation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 25081-01 LCB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Covalent Modification of Hemoglobin in Hydroxyurea Treated Patients

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Arielle Boulet Special Volunteer LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK

COOPERATING UNITS (if any)

Department of Biochemistry, Rockefeller University (J. Manning), Division of Hematology, Cornell University Medical College (B. Weksler)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 0.6

PROFESSIONAL: 0.6

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Recent clinical trials have substantiated hydroxyurea (HU) to be a potent effector of hemoglobin F (HbF) production in patients with sickle cell disease. This augmentation in HbF levels in patients is associated with a decrease in relative beta chain synthesis and thus an inhibitory effect on hemoglobin S (HbS) polymerization both through the decrease in MCH (S)C and the specific sparing effect of HbF. In order to gain insight into the possible genetic mechanisms underlying these effects, we used cation exchange (CE) and reverse-phase high performance liquid chromatography (HPLC) to examine changes in the levels of each hemoglobin species. Samples from ten patients treated with HU were analyzed approximately twice each week. Within the first 50 days of HU treatment, HbF increased on average from 3.3% to 7%. The patients had an average initial ratio of G-gamma/A-gamma of 0.72 which did not change with treatment. At baseline, approximately 15-20% of the fetal hemoglobin exists in the acetylated form (F1) as determined by CE-HPLC, independent of subtype. With HU treatment, the total amount of F0 and F1 increases roughly proportional to the increase in F-reticulocyte numbers. Although the total HbA2 level appears unaffected by treatment, there is an increase in the proportion of one modified HbS species, which may co-migrate with Hb A2, after about 50 days of treatment. For three patients treated with a combination HU and erythropoietin, we found a further increase in both F1 and this modified HbS fraction to levels approaching 5% and 10% respectively.

Subsequent analysis of this modified beta-globin polypeptide showed that the NH2 was not blocked (Edman sequencing) and that the modifier had a mass of 300 close to the one of glutathione (307) (mass spectroscopic analysis). A dual strategy using deblocking and synthetic methods has been used followed by IEF analysis and confirmed the glutathione nature of the adduct. Chemically modified HbS may interfere with HbS polymerization and thus have an added clinical benefit. Acetylation of other proteins (e.g. histones) may be involved in the regulation of gene expression; naturally occurring glutathione adducts have only been reported very recently. It is possible that their formation is related to metabolic effects of HU treatment or, as with acetylation, this modification may be indicative of induced fetal erythropoiesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 25082-01 LCB
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Erythropoietin Receptor: Transcriptional Control		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> <div> PI: Kyung Chin Others: Constance Tom Noguchi Yongji Wu </div> <div> Microbiologist Research Physicist Special Volunteer </div> <div> LCB, NIDDK LCB, NIDDK LCB, NIDDK </div> </div>		
COOPERATING UNITS (If any) Beijing Medical College, Beijing, China (Dr. Y. Wu)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, MD		
TOTAL STAFF YEARS 1.4	PROFESSIONAL: 1.4	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div> <input type="checkbox"/> (b) Human tissues </div> <div> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) <p>The protein, erythropoietin, is a primary regulator of erythropoiesis. The binding of erythropoietin to the surface of hematopoietic progenitor cells is followed by receptor mediated endocytosis of erythropoietin. We have recently cloned the human erythropoietin gene which extends 2 Kb 5' of the canonical start site. The 5' flanking region does not contain TATA or CCAAT sequences usually associated with proximal promoters of cellular genes. The minimal promoter contains an SP1 binding consensus sequence located at minus 19 in addition to the binding consensus sequence for the erythroid specific transcription factor, GATA-1, located at -47.</p> <p>In gel mobility assays, nuclear extracts from erythroid and non-erythroid cell lines provide similar protein-DNA binding patterns to the proximal promoter. An additional band was observed in erythroid cell lines, likely corresponding to GATA-1 binding. In DNase I footprinting, we observed extensive protein binding to the region about -19, the location of the SP1 site, and the GATA-1 binding site at -47. Other protein binding regions are also detected within the proximal promoter including AP2 and another region with no known consensus sequences.</p> <p>Truncated deletion mutations of the regions extending beyond the cap site were linked to a luciferase reporter gene and assayed in OCIM1 erythroid cells by transient transfection. Deleting the unknown footprinted region at -151 in the promoter fragment resulted in an increase of activity to about 1.8 of the activity observed with the longer construct extending to 200 bp 5' indicating that the unknown footprint region may have a negative regulatory effect on the erythropoietin receptor promoter. Deleting the AP2 site reduced transcriptional activity to levels observed with the reference fragment indicating that the AP2 site may have an enhancing activity or a positive regulatory effect on the promoter activity. Surprisingly, deletion of the GATA-1 site at -47 in a promoter fragment reduced the transcriptional activity to only about 80% of the reference sequence. This suggests that while the GATA-1 site is able to enhance activity of the promoter, it is not absolutely required for promoter activity. This construct extending to only -29 leaves an SP1 site at -19 intact, suggesting that the SP1 site is sufficient to obtain at least minimal promoter activity. In contrast, deletion of these 5' sequences to +3 of the cap site markedly reduces luciferase activity to about 20% of the reference construct. These results suggest that SP1 is required for promoter activity and that GATA1 acts to further activate transcription.</p>		

ANNUAL REPORT OF THE LABORATORY OF CHEMICAL PHYSICS NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research in the Laboratory of Chemical Physics is primarily concerned with problems in structural biology and biophysics. There are direct applications of much of this work to the pathophysiology and treatment of human diseases such as AIDS, malaria, and sickle cell anemia. A variety of structural and spectroscopic techniques are employed in these investigations, including nuclear magnetic resonance (NMR), Raman and infrared spectroscopies, time-resolved optical spectroscopy with nanosecond and picosecond lasers, and non-linear optical spectroscopy. There is also a major effort in theoretical studies to complement the experimental work, including both analytical methods and the use of high speed computers in large scale calculations. The systems under study include small prototypical molecules, nucleic acids, proteins, intact and model membranes and retinal photoreceptors. Current research focusses on : the development and application of new NMR methods to the determination of the three-dimensional structure of polypeptides and proteins in solution, conformational and dynamical properties of membrane systems, dynamics of ligand binding and conformational changes in proteins, theoretical analysis of kinetics and chemical dynamics, computer simulations of protein motions, the analysis of excited electronic states of polyenes, the molecular mechanism of excitation in photoreceptor cells and ionic processes in cell membranes, the gelation of hemoglobin S and its relation to the pathophysiology and therapy of sickle cell disease, and the synthesis of potential therapeutic agents for malaria.

Bax and colleagues continue to make major advances in the development of new methods for the determination of the three-dimensional structure of proteins in solution. In a recent analysis of citations of papers published in chemical journals during the period 1984-1990, Bax's papers were cited more than any other scientist in the world (an average of 48 citations per paper for 64 papers). During the past year Bax and colleagues have developed new methods for the measurement of a variety of J couplings in proteins which give information on the backbone conformation. They have also developed a method for studying the structure of a ligand bound to a protein, based on the suppression of all signals of protons that are attached to ^{13}C and ^{15}N nuclei of the uniformly labelled protein. This method was used to show that a 26 residue peptide, M13, that is a random coil when free in solution, forms an α -helical structure in a complex with calmodulin. Bax and colleagues have also studied the dynamics of calmodulin. These measurements show that motional anisotropy of the individual globular domains is three-fold smaller than the values predicted from hydrodynamic calculations for a molecule with a rigid central helix. The internal dynamics of the central helix show that the residues in the middle of this helix act as a flexible linker.

Clare, Gronenborn and colleagues have developed and exploited multi-dimensional NMR techniques to investigate the three-dimensional structures of a large number of proteins in solution. In this work they have demonstrated that it is possible to determine the structure of proteins in solution by NMR with an accuracy comparable to that of high resolution X-ray crystallography. With Davies and Shaanan (LMB) they have also

developed the first method for making detailed comparisons of protein structures in crystal and solution by carrying out a joint refinement of the X-ray and NMR structures to look for genuine differences. Structures studied during the past year include interleukin-4, the calmodulin-target peptide complex (with Bax), ribonuclease H domain of HIV-1 reverse transcriptase, human thioredoxin, the human enhancer binding protein MBP-1, the immunoglobulin binding domain of Streptococcal protein G, and the E3 binding domain of 2-oxoglutarate dehydrogenase. Using a series of 3D double and triple resonance heteronuclear NMR experiments interleukin 4 has been shown to consist of a left-handed four helix bundle with an unusual topology comprising two overhand connections which is remarkably similar to that of growth hormone and granulocyte-macrophage colony stimulating factor, despite the absence of any sequence homology. In the complex of calmodulin with a target peptide made up of the binding domain of myosin light chain kinase the calmodulin domains act to clamp the peptide in a hydrophobic channel; this may be a common binding motif for calmodulin with other peptides. Investigation of the backbone dynamics of the ribonuclease H domain show that it has extensive mobility throughout its structure, particularly at the C-terminus, which has been proposed as the substrate binding site and may explain the lack enzymatic activity of the isolated domain. Knowledge of the structure of thioredoxin has permitted a detailed study of its acid-base titration behavior and has provided structural explanations for the anomalous pK's. Studies of the immunoglobulin binding domain of the Streptococcal G protein show solvent induced distortion α -helices, suggesting that the water molecules are an integral part of the protein structure.

Becker and colleagues have been developing new NMR methods for studying partially-oriented small molecules in liquid crystals. They are also exploring the mechanism and potential applications of the effect of isotopic substitution on NMR chemical shifts of nuclei up to 10 bonds away from the site of substitution.

Szabo and coworkers have carried out several theoretical investigations on the nature and functional significance of a variety of dynamical processes. Computer simulations of reversible, diffusion influenced reactions for simple model reactions have confirmed the analytic theory of Szabo. In particular, the prediction that at long times concentrations relax to equilibrium not exponentially, as expected from simple chemical kinetics, but rather as a power law has been verified. With Clore, Gronenborn, and Brooks (DCRT), Szabo has compared the results of a molecular dynamics simulation of interleukin 1 β in water with those obtained from NMR relaxation measurements. The simulations and experiments suggest a picture in which the N-H bonds in loop regions of the protein undergo large amplitude jumps between conformations stabilized by hydrogen bonds due to infrequent dihedral transitions. With Pastor (FDA) Szabo has developed an analytic expression for the rate of flipping of a Langevin linear rotor in an effective bistable potential, which is the simplest example of a barrier crossing problem that is not one-dimensional. The excellent agreement of the theory with the results of simulations suggests that it should be useful for extracting the viscosity from dielectric measurements on liquid crystals.

During the past year the focus of Zwanzig's research has been on the theory of protein folding and on the role of entropy barriers in determining reaction rates. One

investigation (with Szabo) was a critical analysis of "Levinthal's paradox." The paradox is that a random search through possible conformations of an unfolded protein will take cosmological times to arrive at the native structure. It was shown that this time can be reduced to a biologically significant size merely by including a small bias, of the order of a few kT in favor of locally native conformations. In another investigation it was found that diffusion through geometric bottlenecks could be described with moderate success as diffusion past an entropy barrier instead of the more commonly used potential energy barriers. In a third investigation the rate of passage through a temporally fluctuating bottleneck was carried out as a possible scenario for the motion of ligands through the interior of proteins. The theory predicts a fractional dependence of the rate on viscosity, as is observed experimentally for the motion of oxygen through myoglobin.

Chen has continued to carry out theoretical studies on bioenergetics and kinetics. In one, an approximate analytical formula has been developed (with Szabo) to describe the dequenching of fluorescent probes used to measure the kinetics of cell fusion. A second study has been concerned with the theoretical modelling of the binding of caldesmon and myosin subfragment-1 to actin, which shows that S-1 may still bind to the actin sites that are already covered by a caldesmon molecule, although the binding strength is reduced.

McDiarmid is carrying out optical spectroscopic studies on the electronic structure and conformation of the excited states of small polyatomic molecules that are models for more complex biological molecules. Cyclopentadiene is being studied as a prototype for obtaining fundamental spectroscopic properties of conjugated molecules. Contrary to most transitions in most molecules, the vibronically-induced two-photon resonant transitions to one of the 3p Rydberg states from the ground state are more intense than the analogous allowed transitions. Assignment of these transitions has permitted the empirical estimate of the magnitude of the interstate coupling in the cis and trans geometric conformers.

Levin, Lewis and coworkers are using Raman and infrared spectroscopy to investigate the structural and dynamical properties of both model and intact biological membranes. They are developing a novel vibrational spectroscopic imaging system which combines the spatial resolving power of optical microscopy and the selectivity of infrared and Raman spectroscopy. By simultaneously measuring the optical absorption and resonance Raman spectra of potentiometrically controlled solutions of cytochrome oxidase these investigators have uncovered anticooperative effects between the heme and copper sites. Vibrational spectroscopic studies have been undertaken on model systems to clarify the factors governing the balance of forces leading to an interdigitated chain membrane bilayer morphology. Studies have also continued on the design of synthetic peptide based surfactants to be used in the treatment of respiratory distress syndrome in newborn infants. From Raman spectroscopic temperature profiles the degree and mechanism by which peptides alter membrane disorder and dynamics can be determined. These techniques are also being used to investigate the interaction of bile salts with model membranes. The bile salt ursodeoxycholate is found to induce domains of interdigitated chain lipids within the membrane.

Eaton, Hofrichter and Henry are using time resolved optical spectroscopy in

photodissociation experiments to investigate structure function relations in myoglobin and hemoglobin. These studies have led to the discovery of the protein relaxation subsequent to photodissociation of the myoglobin carbon monoxide complex that may be responsible for a time-dependent geminate rebinding rate; measurements of the viscosity dependence of the rate suggest that at low temperatures conformational substates may not be as much "frozen" as "stuck". Polarized photolysis experiments on myoglobin show that the optical anisotropy prior to rotational diffusion is lower than that predicted for a rigidly attached, perfect circular absorber. Henry has carried out molecular dynamics simulations which suggest that a large part of the decreased anisotropy arises from the reorientational motion of the heme in response to global motions of the surrounding protein. Eaton and Henry (with Mozzarelli, Parma) have continued their investigation of the oxygen binding by single crystals of hemoglobin, and have taken advantage of the different orientations of the individual hemes in the crystal to extract separate binding curves for the alpha and beta subunits from the polarized absorption measurements. This analysis shows that there is very little cooperativity in the crystal, much less inequivalence in the oxygen binding by the alpha and beta subunits than predicted by the analysis of the X-ray data, and no Bohr effect which is consistent with the central role of the salt bridges. Studies by Christoph, Hofrichter, and Eaton on the domain structure and kinetics of hemoglobin S gel formation suggest that homogeneous nucleation is indeed responsible for triggering the formation of a polymer domain, but that each domain contains multiple homogeneously nucleated polymers.

Hagins and Yoshikami are investigating the mechanism of visual transduction. They are also addressing the general problem of introducing known quantities of membrane-impermeable polar substances into isolated cells, tissues, and even whole animals. A series of membrane impermeable fluorescent dyes have been modified by substituting most of their functional groups with a variety of silyl esters or ethers. The silyl derivatives penetrate the lipid membrane rapidly and are then slowly hydrolyzed by the cell water, trapping them inside the cell. The method successfully loads dyes into retinal tissue. Several new techniques are being developed, including the use of a perfusion chamber to hold isolated cells, photometry for measuring changes in intracellular proton and calcium ion concentrations, and improved pyroelectric microcalorimetry.

Ziffer is synthesizing novel compounds that are potential agents for the treatment of malaria. The fungus Beauveria sulfurescens has been used to introduce hydroxyl groups on unactivated methyl, methylene and methine groups of artemisinin derivatives. In a second approach the hydroxy group has been replaced with an allyl group. The most active compound against P. falciparum in vitro, 10 β -propyl-deoxoartemisinin, is currently being evaluated for anti-malarial activity in infected mice. In the course of these studies a new acid catalyzed rearrangement of the peroxide group in dexoartemisinin derivatives was discovered.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29001-20-LCP

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular dynamics and vibrational characteristics of membrane assemblies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Ira W. Levin Research Chemist LCP-NIDDK

Others: E. Neil Lewis Visiting Associate LCP-NIDDK
Paul Harmon IRTA LCP-NIDDK
Patrick Treado IRTA LCP-NIDDK
Michael Batenjany IRTA LCP-NIDDK
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COOPERATING UNITS (If any)

R. Adams, LCP-NIDDK; C. Huang, School of Medicine, Univ. of VA; J.S. Vincent, Univ. of MD; R. Hendler, NHLBI; B. J. Litman, School of Medicine, Univ. of VA; C. G. Cochrane, Research Institute of Scripps Clinic, CA.

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

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TOTAL STAFF YEARS:

5.5

PROFESSIONAL:

5.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A. A novel vibrational spectroscopic imaging system has been designed which combines the spectral resolving power of optical spectroscopy with the exquisite sensitivity and selectivity of vibrational infrared and Raman spectroscopies, techniques involving the absorption and inelastic scattering processes, respectively, of radiation by molecules. The imaging spectrometer, which is entirely solid state, is constructed around an acousto-optic tunable filter (AOTF) coupled to a refractive microscope optimized to the near-infrared or visible spectral regions with either a liquid nitrogen cooled, state-of-the-art, indium antimonide focal plane array detector or a silicon charge-coupled device detector. High fidelity Raman or near-infrared images are produced with unprecedented speeds; the maximum spatial resolution is $\sim 1\mu\text{m}$ and is limited only by diffraction. The instrumentation accommodates both macroscopic and microscopic samples. The data generated by the spectrometer provides a series of 2-dimensional images as a function of wavelength. Since the data can also be viewed as a series of spatially resolved near-infrared or Raman spectra, the conformational and dynamic properties of specific domains within biological or chemical samples can be probed despite the existence of multiple components being present in complex matrices. A variety of model and intact membrane and polymer systems have been examined using either the near-infrared absorption, reflectance or Raman scattering modes of the imaging instrumentation.

B. The oxidation-reduction properties of cytochrome oxidase and related systems have been examined by the simultaneous measurement of the resonance Raman and optical absorption spectra of potentiometrically controlled solutions. Redox data obtained from cytochrome oxidase in its native form argue against the traditional neoclassical models for the enzyme in which the heme a and a₃ centers behave identically and titrate at the same voltages. Specifically, heme a displays effective midpoint potentials near 350 and 220 mV, while heme a₃ exhibits lower E_m 's near 230 and 200 mV. For the cyanide bound complex of cytochrome oxidase, the heme a redox behavior is described by midpoint potentials at 350 and 260 mV. These data are consistent with the notion of anticooperative effects occurring with the heme and copper centers of the enzyme.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DK-29005-18-LCP
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PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Asymmetric Synthesis: Structure, Stereochemistry, and NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.'s	Herman Ziffer	Research Chemist	LCP/NIDDK
Others:	Yuming Pu	Visiting Fellow	LCP/NIDDK
	Juan Rodriguez	Guest Worker	LCP/NIDDK

COOPERATING UNITS (if any)

Dr. Sanford Markey, LCS/NIMH; Dr. H. H. C. Yeh, LAC/NIDDK

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Some 270 million people worldwide are infected with malaria which causes between 1 and 2 million deaths per year. The formidable problems encountered in developing a malaria vaccine, and the ability of Plasmodium falciparum to become resistant to new drugs, has stimulated the World Health Organization and others to search for new drugs to treat this disease. Artemisinin has served as a lead compound to develop new drugs to treat the resistant strains of P. falciparum that have spread over southeast Asia and threaten India and Africa. Since no strains P. falciparum resistant to artemisinin derivatives have appeared, many derivatives (esters and ethers of dihydroartemisinin) have been prepared in China and the United States. Metabolic studies of arteether, a dihydroartemisinin derivative scheduled for clinical tests, found that it is rapidly dealkylated to dihydroartemisinin. It is very likely that esters of dihydroartemisinin are rapidly hydrolyzed to the same compound. In order to prepare compounds that can not be degraded to dihydroartemisinin, we introduced additional functional groups into artemisinin by a microbially mediated oxidation using Beauveria sulfurescens. Derivatives of these hydroxylated materials have been prepared and tested. IN a second approach we replaced by hydroxy group of dihydroartemisinin with an allyl group. The double bond of the allyl substitute enabled us to introduce polar groups into the molecule so as to adjust its lipo- or hydro-philicity. The polarity is important in deciding the mode of administration. Most of the compounds were found to be as or more effective than artemisinin and arteether against P. falciparum in vitro. The best compound, the n-propyl derivative, is undergoing in vivo testing in mice infected with P. berghei.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29006-22-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The structure and dynamics properties of macromolecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Elliot Charney

Scientist Emeritus

LCP-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Spectroscopy and Structure

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

PROJECT HAS BEEN TERMINATED

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29007-20-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and interaction of biomolecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Hideo Kon Research Chemist LCP-NIDDK

COOPERATING UNITS (If any)

LAB/BRANCH

Laboratory of Chemical Physics

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INSTITUTE AND LOCATION

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TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

PROJECT HAS BEEN TERMINATED

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29008-21-LCP

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Electric and molecular structure investigation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Ruth McDiarmid Research Chemist LCP-NIDDK

Others Aharon Gedanken Special Volunteer LCP-NIDDK
Xing-Xing Visiting Fellow LCP-NIDDK

COOPERATING UNITS (if any)

Leo Klasinc and Brank Kovac, Rudger Boscovic Institute, Zagreb, Croatia

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Spectroscopy and Structure

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.9

PROFESSIONAL:

1.9

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The observation and analysis of the true and vibronic false origins of the 3p Rydberg \leftarrow X transitions of cyclopentadiene revealed by polarization-selected two-photon resonant multiphoton ionization spectroscopy has enabled absolute assignments to be made for these transitions.

Based on these assignments, the energies of the transitions, and previously reported or literature values for the energies of the 3p Rydberg \leftarrow X and valence transitions of butadiene and benzene, an empirical determination of the extent of interstate coupling was made for s-cis and s-trans dienes. The results are essentially none for the s-cis conformer and about 25 percent for the s-trans conformer. These results support theoretically calculated values of these interactions.

A new research project on the dynamics of the photochemistry of dienes has been initiated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29010-20-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dynamics of Proteins and Studies on Sickle Cell Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	William A. Eaton	Medical Officer	LCP-NIDDK
Others:	James Hofrichter	Research Chemist	LCP-NIDDK
	Eric R. Henry	Research Chemist	LCP-NIDDK
	Anjum Ansari	Visiting Associate	LCP-NIDDK
	Colleen M. Jones	Staff Fellow	LCP-NIDDK
	Garrott Christoph	Expert	LCP-NIDDK

COOPERATING UNITS (If any)

Andrea Mozzarelli, Institute of Biochemical Sciences, University of Parma, Italy

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Time resolved spectroscopy in photodissociation experiments, polarized single crystal absorption spectroscopy, and the molecular dynamics simulations are being used to investigate structure function relations in myoglobin and hemoglobin. The techniques of high-precision nanosecond spectroscopy in partial photolysis experiments has been developed and has led to several important new findings, including (i) demonstration that the transition state for the quaternary structural change of hemoglobin is much more like the R conformation than the T conformation, a result which explains the linear free energy relation between the quaternary rates and equilibrium constants, (ii) discovery that the protein relaxation subsequent to photodissociation of the myoglobin carbon monoxide complex exhibits a viscosity dependence consistent with Kramer's theory with a modified friction term to account for the internal friction of the protein (iii) demonstration in hemoglobin that there is no communication between subunits prior to the quaternary conformational change at about 10 microseconds, (iv) and confirmation of theoretical predictions for the dependence of the optical anisotropy on the degree of photolysis with linearly-polarized light pulses.

Polarized single crystal absorption measurements show that crystals of haemoglobin in the T quaternary structure bind oxygen noncooperatively with no Bohr effect. These results provide strong evidence for the two-state allosteric model and for Perutz stereochemical mechanism of the Bohr effect.

Studies of the domain structure and kinetics of hemoglobin S gel formation support the hypothesis that homogeneous nucleation of a single polymer molecule can trigger the formation of an entire domain of polymers, and that variations in the rate of homogeneous nucleation can account for the wide variety of shapes for sickled cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29011-21-LCP

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The physics and chemistry of photoreception

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I. William A. Hagins Medical Officer LCP-NIDDK

Others: S. Yoshikami Research Biologists LCP-NIDDK
P. Ross Research Chemist LMP-NIDDK
K. Spring Research Med. Officer LKM-NHI
Mark Vivino Computer Systems Analyst CSL-DCRT
L. Pannell Research Chemist LAC-NIDDK

COOPERATING UNITS (if any)

Sudhir Sahu and Sheila Shah, Student Volunteers, Thomas Jefferson High School, Alexandria, VA

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INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Calcium metabolism in retinal rod outer segments during phototransduction is being studied with the aid of fluorochromic dyes introduced into retinas by new methods and studied by quantitative microscopic image systems.

Calorimetric, X-ray microanalytic and photometric methods are being used to study ionic and biochemical events during phototransduction.

New techniques for breaching the blood-brain barrier to introduce polar molecules into brain and retinal tissue are under study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201-DK-29016-15-LCP

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Macromolecular dynamics and assembly reactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	James Hofrichter	Research Chemist	LCP-NIDDK
Others:	Garrott Christoph	Special Expert	LCP-NIDDK
	William A. Eaton	Medical Officer	LCP-NIDDK
	Eric Henry	Research Chemist	LCP-NIDDK
	Anjum Ansari	Visiting Associate	LCP-NIDDK
	Colleen Jones	Visiting Fellow	LCP-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Laser Biophysics and Spectroscopy

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.1

PROFESSIONAL:

2.1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Time-resolved absorption spectroscopy is used to study the dynamics of protein structural changes subsequent to excitation with short laser pulses. Molecular models for the protein dynamics are used to fit and interpret the measured data.

A. The kinetics of ligand binding and conformational changes for sperm whale myoglobin and human hemoglobin (HbA) have been studied following the set of data from partial photolysis experiments using polarized excitation has permitted cooperative and noncooperative processes to be clearly distinguished. We are now proceeding to test specific molecular models for the rebinding dynamics.

B. The photocycles of bacteriorhodopsin (bR), a photoactive proton pump have been investigated. A detailed analysis of data collected by Xie et al. has shown that back reactions are significant for all steps of the cycle except the last. The temperature- and pH-dependence of these rates is obtained from fits of the model to the data.

C. The effect of polymerization kinetics on the morphology of gels of hemoglobin S. has been investigated by polarizing microscopy and computer simulations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29017-13-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Spectroscopic investigation of membrane lipids and models

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Ralph G. Adams Research Physicist LCP-NIDDK

Others: Ira W. Levin Research Physicist LCP-NIDDK

COOPERATING UNITS (if any)

Sherwin Strauss (FDA)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Using a variety of spectroscopic techniques, we have investigated the role of phospholipid, principally dipalmitoylphosphatidylcholine (DPPC), in respiration. Because of the high content of protein there is a significant fluorescence associated with pulmonary surfactants. Since dispersive Raman spectroscopic techniques using visible laser excitation cannot be applied to highly fluorescing samples, we have used the new Fourier transform (FT) Raman approach coupled with near-infrared laser excitation. This spectroscopic technique allows one not only to obtain a Raman spectrum, but to determine spectral peak frequencies with high precision.

Progress has been made in clarifying the role of the various protein proteins of native lung surfactant, but no clear, unequivocal picture has yet been forthcoming, here or elsewhere. We are still pursuing that goal.

We have, however, established FT Raman spectroscopy as a practical tool for sorting out suitable lung surfactant substitutes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29019-12-LCP

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Theoretical studies on the dynamical aspects of macromolecular function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. A. Szabo Research Chemist LCP-NIDDK

Others: X. Zhou Visiting Associate LCP-NIDDK

COOPERATING UNITS (if any)

R. Zwanzig, A. Gronenborn, G. Clore, and B. Bagchi -(all) LCP-NIDDK; R. Pastor, FDA; I. Chandrasekhar, and B. Brooks - (all) DCRT; P. Richards, Sandia National Laboratory

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Theoretical Biophysical Chemistry Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Several theories for the kinetics of reversible diffusion influenced bimolecular reactions, developed in this laboratory, have been evaluated by comparison with exact results obtained via computer simulations of simple model reactions. No adjustable parameters were used and the good agreement between theory and simulations was found. In particular, the prediction that at long times concentrations relax to equilibrium not exponentially, as expected from simple chemical kinetics, but rather as a power law, has been verified. An analysis of a simple model of protein folding showed that Levinthal's astronomical folding time (10(to the power of 30)years) is reduced to a biologically relevant size (seconds) if correct local conformations are assumed to be more stable than incorrect ones by as little as 1kcal/mol. Computer simulations of u152 amino acid protein in water suggested a molecular picture of the slow (00ps-1ns) motions of N-H bonds that has been inferred from NMR relaxation experiments: the N-H groups of residues in loop regions of the protein undergo large amplitude jumps between conformations stabilized by hydrogen bonds due to infrequent dihedral transitions. The langevin dynamics of a linear molecular in a liquid crystal was simulated in order to test an analytical expression for the flipping rate. The nature of dielectric and orientational relaxation in a brownian dipolar lattice was analyzed using computer simulations and it was found that these relaxations become significantly nonexponential with increasing polarity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29020-08-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nuclear magnetic resonance: new methods and molecular structure determination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Ad Bax Visiting Scientist LCP-NIDDK
Others Rolf Tschudin, Electronics Engineer; Mitsuhiro Ikura, Visiting Associate;
Lewis Kay, Special Volunteer; Frank Delaglio, Special Expert; Jacob Anglister,
Visiting Scientist; Geerten Vuister, Visiting Fellow, Stephan Grzesiek, Visiting
Associate; David Live, Special Volunteer; Guang Zhu, Student Volunteer; Andrew Cox,
Student Volunteer - all LCP/NIDDK

COOPERATING UNITS (If any)

Marius Clore, Angela Gronenborn, LCP-NIDDK; Dennis Torchia, LBR-NIDR; Claude Klee,
LC-NCI

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Biophysical Nuclear Magnetic Resonance Spectroscopy Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

8.0

PROFESSIONAL:

8.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

NMR methods have been developed that facilitate the resonance assignment for proteins that can be isotopically enriched with carbon-13 and nitrogen-15 for the study of unlabeled ligands complexed with the uniformly labeled protein.

Application of these new methods to the protein calmodulin, in the absence and presence of an unlabeled peptide fragment of skeletal muscle myosin light chain kinase, reveal pronounced structural differences in calmodulin. In the presence of calcium and in the absence of peptide, calmodulin consists of two globular domains, connected by a flexible linker. Nitrogen-15 relaxation studies indicate that the two globular domains reorient nearly isotopically, in contrast to what would be expected for the structure observed in the crystalline state where the two domains are connected by a long alpha-helix (the so-called "central helix"). In the presence of peptide, the relative orientation of the two calmodulin domains is well determined. The peptide-protein complex adopts an approximately ellipsoidal shape, with the peptide in an alpha-helical conformation clamped in between the two domains. The structure calculated to date are based on only a fraction of the spectral information available from the 3D and 4D NMR spectra. Work is currently in progress to refine the structure by adding more NOE and J-coupling constraints.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29021-07-LCP

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Conformation and dynamics of biological macromolecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Eric Henry	Research Physicist	LCP-NIDDK
Others:	William Eaton	Medical Officer	LCP-NIDDK
	James Hofrichter	Research Chemist	LCP-NIDDK
	Anjum Ansari	Visiting Associate	LCP-NIDDK
	Colleen Jones	Staff Fellow	LCP-NIDDK
	Attila Szabo	Research Chemist	LCP-NIDDK

COOPERATING UNITS (if any)

Andrea Mozzarelli, Institute of Biochemical Sciences, University of Parma, Italy

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Biophysical Chemistry Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have determined oxygen binding curves from polarized optical absorption spectra of single crystals of human hemoglobin measured as a function of oxygen pressure. An analysis of the oxygen binding properties of the alpha and beta subunits in the crystal indicates that the observed Hill n close to 1.0 may arise from a compensation effect between inequivalent oxygen binding to the two types of subunits and a degree of cooperativity in the binding to the protein in the T quaternary structure. We have also studied the effects of photoselection on time-resolved spectra measured in partial-photolysis experiments using linearly polarized excitation and probe pulses. The ability to obtain isotropically averaged spectra which are free of these effects has allowed the determination of the conformational and ligand binding kinetics of myoglobin and hemoglobin with unprecedented accuracy. We have also performed long molecular dynamics simulations to study heme reorientational motions in myoglobin. In these simulations the heme orientation and the protein backbone conformation explore different regions of their respective spaces in a correlated fashion. The simulations predict subnanosecond heme reorientational motions which account for about 30 percent of the reduction of the initial absorption anisotropy observed in partial photolysis experiments on myoglobin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29022-LCP

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural Studies of AIDS proteins by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.'S	Angela Gronenborn	Visiting Scientist	LCP-NIDDK
	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Ad Bax	Visiting Scientist	LCP-NIDDK
Others	Daniel Garrett	IRTA Fellow	LCP-NIDDK
	Jim Omichinski	Staff Fellow	LCP-NIDDK
	Robert Powers	IRTA Fellow	LCP-NIDDK

COOPERATING UNITS (if any)

Boaz Shaanan, David Davies - LMB-NIDDK; Stephen Stahl, Paul Wingfield - Protein Expression Laboratory; Ettore Appella, K. Sakaguchi - DCBD-NCI; Bernard Brooks, Indira Changrasekhar - DCRT; Attila Szabo - LCP-NIDDK; Carl March - IMMUNEX

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Sections on Structural Biology, Protein NMR, and Biophysical NMR Spectroscopy

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Work has been carried out on number of structural problems related to proteins derived from the HIV virus. These include the RNase H domain of HIV-1 reverse transcriptase, the p7 nucleocapsid protein of HIV-1, and proteins of the immune system, in particular interleukin-1beta, interleukin-4 and the double zinc finger of the human enhancer binding protein MBP-1. The solution dynamics of the RNase H domain has been analyzed using (superscript) 1H -(superscript)15N heteronuclear NMR spectroscopy. The solution structure of interleukin-4 and the double zinc finger domain of the human enhancer binding protein NMP-1 (which binds specifically to the regulatory region in the long terminal repeat of the HIV genome) have been determined. The RNA binding properties of the p7 nucleocapsid protein have been investigated as a prelude to structural studies of specific p7-RNA complex. The structure of interleukin-1beta has been further investigated using a new method involving the simultaneous and combined use of NMR and crystallographic data in the refinement process.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29023-LCP

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Determination of Three-Dimensional Structures of Macromolecules in Solution by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK
Other:	Julie Forman-Kay	IRTA Fellow	LCP-NIDDK
	Daniel Garrett	IRTA Fellow	LCP-NIDDK
	Bruce Grasberger	IRTA Fellow	LCP-NIDDK
	Patricia Lodi	Guest Researcher	LCP-NIDDK
	Jim Omichinski	Staff Fellow	LCP-NIDDK

COOPERATING UNITS (If any)

A. Bax and A. Szabo -LCP-NIDDK; B. Shaanan, D. Davies, G. Felsenfeld - LMP-NIDDK;
S. Stahl, P. Wingfield - Protein Expression Laboratory; E. Appella, C. Klee - NCI;
B. Brooks, I. Chandrasekhar - DCRT; M. Whitlow - Enzon, Inc.; C. March - Immunex

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Sections on Protein NMR and Structural Biology

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Work in this laboratory has been focussed on the determination of three dimensional structures of larger proteins in solution by NMR, with a particular emphasis on cytokines and immune related proteins. A considerable effort has been placed on the development of three- and four-dimensional heteronuclear NMR to extend the application of NMR as a method for determining three-dimensional structures of proteins in solution beyond the limits of conventional two-dimensional NMR (approximately 100 residues) to molecules in the 150- to 400- residue range. In addition, a novel approach to structure determination has been developed which involves combining data from both NMR and X-ray crystallography simultaneously in the refinement process.

High resolution solution structures of a number of proteins have been determined. These include the cytokine interleukin-4, the complex of calmodulin with a target peptide from skeletal muscle myosin light chain kinase, the E3 binding domain of the dihydrolipoamide succinyl transferase core from the 2-oxoglutarate dehydrogenase multienzyme of *E. coli*, and the double CYS (subscript)2His(subscript)2 zinc finger from the human enhancer binding protein MBP-1. Extensive use in these studies has been made of multi-dimensional heteronuclear NMR and of systematic conformational searches to obtain stereospecific assignments and torsion angle restraints which have enabled us to obtain very high resolution structures comparable in accuracy to 2 Å resolution X-ray structures. The typical accuracy attainable is 0.2-2.4 Å for the backbone atoms and 0.4-0.5 Å for the internal side chains.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29025-03-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Investigations of Macromolecular Structures and Dynamics in Solution by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.'S Angela M. Gronenborn Visiting Scientist LCP-NIDDK
 G. Marius Clore Visiting Scientist LCP-NIDDK

Others: Julie Forman-Kay, Daniel Garrett, Bruce Grassberger, Robert Powers:
 IRTA Fellows, LCP-NIDDK; Patricia Lodi - Guest Researcher, LCP-NIDDK; Jim
 Omichinski - Staff Fellow, LCP-NIDDK; Mark Robien - Howard Hughes Fellow; Teresa
 Strzelecka, Visiting Associate, LCP-NIDDK

COOPERATING UNITS (if any)

Ad Bax and Attila Szabo, LCP-NIDDK; Boaz Shaanan, David Davies, Gary Felsenfeld -
LMB-NIDDK; Stephen Stahl, Paul Wingfield, Protein Expression Laboratory; Ettore
Appella, Claude Klee, NCI; Bernard Brooks, Indira Chadraseskhar, DCRT

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Sections on Structural Biology and Protein Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The objective of the overall research in this laboratory is centered on achieving as complete a description as possible for the structures of peptides, proteins, nucleic acids and their complexes in solution, principally by NMR spectroscopy. At present particular emphasis is being placed on developing approaches which allow the investigation of larger and more complex systems, and increases the precision with which these solution structures can be obtained, as well as on studies aimed at correlating structure and function.

Structures for several proteins have been determined and analyzed. These include the cytokine interleukin-4, the complex of calmodulin with a target peptide from skeletal muscle myosin light chain kinase, the double zinc finger domain of the human enhancer binding protein MBP-1, and the E3 binding domain of the dihydrolipoamide succinyl transferase core from the 2-oxoglutarate dehydrogenase multienzyme complex of (*italic*) E. coli. These studies have exploited many novel 3D and 4D heteronuclear NMR experiments to dramatically increase spectral resolution and thereby resolve assignment ambiguities in larger proteins. The location and effect of structural water in the IgG binding domain of streptococcal protein G has been investigated and has provided the first demonstration of water induced distortion of a helix in solution. Finally, a detailed analysis of the electrostatic properties of human thioredoxin has been carried out and related to its structure.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29026-04-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

NMR and other spectroscopic studies of molecular structure

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Edwin D. Becker Research Chemist LCP-NIDDK

Others: Drazen Vikić-Topić Visiting Associate LAC-NIDDK

COOPERATING UNITS (if any)

Sophisticated Instruments Facility, Indian Institute of Science, Bangalore, India; Centre for Cellular and Molecular Biology, Hyderabad, India; Molecular Graphics and Simulation Laboratory, DCRT

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.7

PROFESSIONAL:

1.7

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

- A. Collaborative studies with C. L. Khetrpal and others at the Indian Institute of Science use nuclear magnetic resonance (NMR) methods to study molecules oriented in liquid crystals. Because the spectra rapidly become very complex with increasing numbers of interacting hydrogen atoms, only small or highly symmetric molecules have been studied. We are exploring a new method to use solid-state NMR spinning techniques to extend the range of applicability of the method.
- B. We are exploring the mechanism and potential applications of the effect of isotopic substitution on NMR chemical shifts, as shown in carbon-13 nuclei up to 10 bonds away from the site of substitution. Ab initio quantum mechanical calculations show that these effects correlate with electron density variations on the carbon atoms caused by the small difference in bond lengths of C-H and C-D bonds.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29027-04-LCP

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Theoretical studies of dynamical processes in chemical physics and biophysics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Robert Zwanzig

Research Chemist

LCP-NIDDK

Others: Biman Bagchi

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Theoretical Biophysics Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The main foci of research have been on the theory of protein folding and on the role of entropy barriers in determining reaction rates. One investigation was a critical analysis of "Levinthal's paradox". The paradox is that a random search through possible conformations of an unfolded protein will take cosmological times to arrive at the native structure. It was shown that this time can be reduced to a biologically significant size merely by including a small bias, of the order of a few kT, in factor of locally native conformations. Another investigation dealt with diffusion through geometrical bottlenecks. It was found that this could be described with moderate success as diffusion past entropy barriers instead of the more commonly used potential energy barriers. A third investigation was motivated by experimental observations on the viscosity dependence of ligand binding to myoglobin. It was found that a temporally fluctuating entropy bottleneck can explain some aspects of these experiments.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DK-29028-02-LCP
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PERIOD COVERED October 1991 through September 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Free energy conversion in biology
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PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)			
P.I.:	Yi-der Chen	Research Chemist	LCP-NIDDK
Others:	Robert J. Rubin	Special Volunteer	LCP-NIDDK

COOPERATING UNITS (if any) Joe Chalovich, Dept. of Biochemistry, East Carolina University School of Medicine; Bernard Breenen, University of Ulm, Ulm, Germany
--

LAB/BRANCH Laboratory of Chemical Physics
--

SECTION Theoretical Biophysics Section

INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, MD 20892
--

TOTAL STAFF YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:
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CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
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SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.) <p>A number of different topics have been studied in the general field of free energy transduction and biophysics of biological systems. The most important areas in which progress has been made are the study of dequenching kinetics of fluorescence of lipid-like probes in membrane fusion systems, equilibrium binding of caldesmon molecules to actin in the presence of myosin molecules, and model study on the transient recovery of force in muscle after a length perturbation.</p>
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ANNUAL REPORT OF THE LABORATORY OF BIOORGANIC CHEMISTRY

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research of the Laboratory is directed towards the introduction of new concepts, techniques and agents for the elucidation of the molecular nature of mechanisms controlling cell functions. Specific focus is placed on i) Development of selective agonists/antagonists for receptors controlling cyclic nucleotide formation, phospholipid metabolism and ion channel function; ii) The relationship between ion transport, phospholipid turnover and cyclic nucleotide generation and the delineation of agents with specific effects on macromolecules involved in these systems. iii) The isolation and structure elucidation of biologically active natural products and definition of the basis of their activity. iv) Effects of agents on ion channels and the development of radioactive ligands for modulatory sites in such channels. v) The nature of enzymes involved in formation and inactivation of neurotransmitters, hormones, and other modulatory substances, in particular the enzymes, catechol-O-methyltransferase, monoamine oxidase, adenylate cyclase and phosphodiesterases. vi) the fundamental mechanisms by which drugs and environmental chemicals are transformed in the body with emphasis on oxidative metabolism by cytochrome P-450 systems to generate active oxide metabolites that interact with macromolecules such as DNA and are metabolized by further oxidation, by hydrolysis and by conjugation with glutathione.

Some of the milestones for the Laboratory are as follows: i) Introduction of the adenine-prelabeling technique for study of cyclic AMP generation in intact cells; ii) The steroidal alkaloid batrachotoxin as a selective activator of sodium channels. iii) Histronicotoxin as a noncompetitive blocker of acetylcholine receptor channels and potassium channels. iv) Pumiliotoxins as myotonic and cardiotonic alkaloids acting through sodium channels to elicit phosphoinositide turnover. v) N⁶-Substituted adenosines and 8-phenyl and 8-cyclohexylxanthines and other heterocycles as selective and potent adenosine receptor agonists and antagonists suitable as radioligands for binding studies and for definition of A₁ and A₂ classes of receptors. vi) Introduction of forskolin as a specific and widely useful activator of adenylate cyclase. vii) Fluoronorepinephrines and analogs as selective alpha and beta-adrenergic agonists. viii) Production of antibodies to catechol-O-methyl transferase and their use in studying localization of this key catechol-metabolizing enzyme. ix) Development of a cyclic quaternary amine, isocarcotolone methiodide, with high potency and selectivity for nicotinic receptors. x) Definition of a relationship between receptor-activation of phosphoinositide breakdown; protein kinase C activation, and altered responses of cyclic AMP-generating systems. xi) Introduction of maitoxin as a general activator for phosphoinositide breakdown. xii) Discovery of the NIH shift of aryl substituents during P-450 catalyzed phenol formation and demonstration of arene oxides as intermediates. xiii) Demonstration of oxidation-hydrolysis pathways that convert stereoselectively polycyclic aromatic hydrocarbons to ultimate diol epoxides that react with DNA. xiv) Discovery and formulation of the bay-region theory, which is predictive of the pathway for formation of reactive carcinogenic metabolites from polycyclic aromatic hydrocarbons. xv) Development of optical assays for protease and reverse transcriptase of HIV-1.

The laboratory accomplishes its mission both through its own resources and

through extensive collaborations with other laboratories both at NIH, at Universities, Museums, and other institutes and in drug and chemical companies. Such collaborations can involve sharing of expertise on syntheses, isolations, analyses and biological testing and field work to obtain sources of new natural products.

SECTION ON PHARMACODYNAMICS

Pharmacologically Active Compounds from Amphibians and Other Natural Sources

Structure elucidation of alkaloids from skin of frogs and toads continues to reveal unique new compounds many with high biological activity. Syntheses for several of these are under investigation, including the pseudophrynamines, which based on structural analogy to physostigmine, may be acetylcholine esterase inhibitors, epibatidine, a novel pyridyl-nortropene with potent non-opioid analgetic activity, the pyrrolidine oximes and a proposed class of dehydro-homopumiliotoxins.

Homobatrachotoxin in Birds. The first example of toxicity and chemical defense in birds was discovered. Three species of birds of the New Guinean genus *Pitohui* possess the steroidal alkaloid homobatrachotoxin, a toxin previously considered unique to neotropical poison-dart frogs of the genus *Phylllobates*. The birds also contain an alkaloid in muscle with local anesthetic activity.

Alkaloid Profiles: Genetic or Environmental Determinants. Dendrobatid frogs produce a diverse set of alkaloids, whose profiles appear characteristic of frogs of each species or, in the case of variable species, of each population. In the case of one widespread species, *Dendrobates auratus*, alkaloid profiles in extracts of skin are markedly different in three populations, one from a Panamanian Pacific Island, one from central mountains in Panama, and the third from the Caribbean coast in Costa Rica. The first contains three major classes of dendrobatid alkaloids, the histrionicotoxins, the pumiliotoxin-A class and the decahydroquinolines. The second contains mainly histrionicotoxins, pumiliotoxin-A class alkaloids and one indolizidine. The third contains histrionicotoxins, a homopumiliotoxin, one decahydroquinoline, and a variety of indolizidines, quinolizidines and pyrrolizidines. Frogs were introduced into the Hawaii, in 1932. Remarkably, although alkaloids of the pumiliotoxin-A class and one decahydroquinoline are still major constituents in skin extracts of Hawaiian frogs descended from the 1932 founding population, histrionicotoxins are absent and a novel tricyclic alkaloid is present. Offspring of wild-caught parents raised in indoor terrariums on a diet of crickets and fruit flies do not contain detectable amounts of skin alkaloids. Offspring raised in large outside terrariums in Hawaii and fed mainly wild-caught termites and fruit flies do contain the same profile of alkaloids as their wild-caught parents in Hawaii, but at reduced levels. The genetic, environmental and dietary determinants of alkaloid profiles in dendrobatid frogs remain obscure, in particular the underlying cause for total absence in terrarium-reared frogs.

Epibatidine: A Novel Chloropyridylazabicycloheptane. A potent non-opioid analgesic, epibatidine, has been isolated from skins of the Ecuadoran poison frog, *Epipedobates tricolor*, and its structure determined by mass spectrometry, and infrared and ¹H-NMR analyses as exo-2-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane. It represents a unique new class of alkaloids and is many fold more potent than morphine as an

analgesic. Its effects are not blocked by the opioid antagonist naloxone. A synthetic route has been developed.

Pyrrolizidine Oximes. Analysis of gas chromatographic infrared spectra and reexamination of ^1H - and ^{13}C -NMR data led to revised structures for three closely related tricyclic alkaloids from a dendrobatid poison-frog *Dendrobates pumilio*. The simplest member, 222, is a spiropentano-pyrrolizidine oxime, while 236 is the corresponding O-methyl oxime and 252, a hydroxy-O-methyl oxime. The O-methyloxime has been synthesized and additional amounts are being prepared for biological investigation.

Alkaloids in Nondendrobatid Frogs. Brightly colored ranid frogs of the Madagascan genus *Mantella* contain a variety of skin alkaloids based on mass spectral and infrared gas chromatographic analysis. All contained one or more representatives of the pumiliotoxin-A class with the 13,14-dihydro derivatives often found in major amounts. Major amounts of two 1,4-disubstituted quinolizidines 217A and 231A and a 5,8-disubstituted indolizidine 217B were found in one species, in addition to many minor or trace quinolizidines and indolizidines. Such quinolizidines and indolizidines were present as trace alkaloids in the six other species of *Mantella*, along with 3,5-disubstituted indolizidines and pyrrolizidines, the decahydroquinoline cis-195A, tricyclic alkaloids and homopumiliotoxins. A new alkaloid class, which appears to contain a quinolizidine moiety, is seen in two species and is represented by 235C and several congeners. Skins of bufonid toads of the genus *Melanophryniscus* contain several classes of alkaloids: Decahydroquinolines, pumiliotoxins, allopumiliotoxins, homopumiliotoxins, both 3,5- and 5,8-disubstituted indolizidines, 3,5-disubstituted pyrrolizidines and a 1,4-disubstituted quinolizidine. Tricyclic alkaloids, including precoccinelline and alkaloid 236, a pyrrolizidine oxime methyl ether, are present in one population of *Melanophryniscus stelzneri*.

Anticonvulsants and batrachotoxin binding. A series of 1-phenylcycloalkane carboxylic acid derivatives of the anticonvulsant carbapentane (CBP) were examined as inhibitors of the specific binding of batrachotoxinin- $[\text{^3H}]$ benzoate ($[\text{^3H}]$ BTX-B) to sodium channels in synaptoneurosome. Anticonvulsant potency was determined by the maximal electroshock technique. Expansion of the pentane ring to a cyclohexane ring with either an amide, ester, or ether modification of CBP resulted in a higher affinity for the BTX site but lower anticonvulsant activity. Replacing the ester with an ether moiety in CBP resulted in a 2-fold increase in anticonvulsant potency with only a minimal decrease in affinity for the BTX site. However replacing the ester with a methylamine linkage in CBP resulted in a complete loss of anticonvulsant activity and only a modest decrease in affinity for the BTX site. There, thus, was no correlation between $[\text{^3H}]$ BTX-B binding and anticonvulsant potency.

SECTION ON PHARMACODYNAMICS

Pharmacology and Metabolism of Biogenic Amines and Related Compounds

COMT:

Light and electron-microscopic immunochemical observations of the localization of the soluble form of COMT in rat uterus and kidney were made with

a specific antibody using the peroxidase-antiperoxidase technique with peroxidase conjugated with streptavidin.

The previous demonstration of the progesterone-dominance in the early appearance of COMT in the luminal epithelium and glandular epithelium of the metrial gland in the uterus of the pregnant and pseudopregnant rat has been extended to include an examination of the effects of antagonists of progesterone receptors on the appearance of COMT. Administration of RU486 on day 0 and day 1 of pregnancy or pseudopregnancy appears to completely block the expected appearance of luminal COMT on day 3. Preliminary results suggests that the absence of luminal COMT leads to a failure of successful implantation in pregnant rats.

COMT and rat macrophage-specific antigens were localized in the corpus lutea of the rat by double immunocytochemical staining. A polyvalent rabbit antisera for the soluble form of rat COMT and a macrophage-specific monoclonal antibody, ED1, which recognizes rat monocytes, macrophages, and dendritic cells were used in this study. Immunoglobulin-gold particles were used to localize the macrophage-specific antibody at the electron-microscopic level. The results clearly demonstrate the coexistence of COMT and ED1 monoclonal antibody positive-reaction products in the same cells in the corpus lutea of the rat ovary and provide convincing evidence the COMT-positive cells are macrophages. We have also demonstrated that macrophages in cervical lymph nodes are the cellular site of both the extraneuronal uptake of norepinephrine and COMT. The extraneuronal site of norepinephrine uptake was determined by histochemical fluorescence. A pronounced granular green fluorescence was present in the cytoplasm of macrophages following norepinephrine uptake. Our results suggest that macrophages provide a model of the extraneuronal transport system for norepinephrine.

The study of COMT activity in hamster kidney following estrogen-induced renal carcinogenesis is still in progress. The distribution of COMT in the hamster kidney is qualitatively similar to that in the rat in that the major site of COMT is in the epithelial cells of the proximal tubules with lesser amounts in the epithelial cells of the collecting ducts. Preliminary results suggests that the level of COMT activity in estrogen-induced hamster renal carcinoma is elevated. The localization and level of COMT in estrogen receptor-positive and -negative endometrial and breast adenocarcinoma is still under study.

Studies on the role of COMT in the biosynthesis of mammalian alkaloids has been extended to an examination of the nature of the additional products formed during the O-methylation of both (R)- and (S)-3'-Hydroxycoclaurine. O-Methylation of 3'-hydroxycoclaurine yielded the expected O-methylethers, N-norreticuline and nororientaline in approximately equal amounts for the (S)- and (R)-antipodes. The additional products derived from 3'-hydroxycoclaurine have been tentatively identified berbines. Chromatographic identity suggests that norreticuline gives rise to scoulerine and coreximine and the nororientaline give rise to 10-demethyliscentrine and stepholidine. Exclusion of exogenous formaldehyde in the O-methylation procedure failed to prevent berbine formation.

Fluorine and Other Derivatives of Biogenic Amines and Amino Acids:

The tissue distribution and time course of incorporation into acid insoluble (bound) and acid soluble (free) fractions of [³H]-fluorohistidine is

compared to that of $U[^{14}\text{C}]\text{Histidine}$ in mouse tissues *in vivo*. The cycloheximide-sensitive incorporation of 2-FHis is between 9 and 17 percent of that of His. Unlike, $[^{14}\text{C}]\text{His}$ a major fraction, approximately 90% at 72 hrs, of isotope derived from $[^3\text{H}]\text{2-FHis}$ remains in tissues for a prolonged period in an acid soluble form. The excretion of isotope derived from $[^{14}\text{C}]\text{His}$ ($T_{1/2} = 5$ hr) is more rapid than from $[^3\text{H}]\text{2-FHis}$ ($T_{1/2} = 11.4$ hrs). 2-FHis, at doses from 100 to 250 mg/kg produce a reversible inhibition of growth in mice.

SECTION ON PHARMACODYNAMICS

Ion Channels, Receptors and Second Messengers in the Nervous System

Maitotoxin: Site of Action. Maitotoxin appears to stimulate calcium influx via a so-called receptor-mediated calcium entry system, thereby causing stimulation of phosphoinositide breakdown. In glioma C6, pheochromocytoma PC12, insulinoma HIT and human blood cells maitotoxin elicits a dose-dependent stimulation of Ca^{2+} influx while having no effect in liposomes. Thus, maitotoxin does not act as an ionophore. In HIT cells maitotoxin also elicited influx of $\text{Rb}^+ > \text{Na}^+ > \text{Mn}^{2+}$, but the stimulation was far less than for Ca^{2+} . Stimulation of Ca^{2+} influx was blocked by Ni^{2+} , Co^{2+} , Cd^{2+} and Mn^{2+} , and markedly reduced by Ba^{2+} . Divalent cations, in particular Ca^{2+} , Ba^{2+} , Mn^{2+} and Cd^{2+} , enhanced influx of the monovalent cations Na^+ and Rb^+ . Partial structures of maitotoxin indicate the presence of double bonds at both termini of this ladder-shaped terpene and a primary alcohol at one terminus. These functionalities were used for preparation of radioligands of MTX namely $[^3\text{H}]\text{perhydro-MTX}$ and $[^3\text{H}]\text{benzoyl-MTX}$. Both radioligands had high levels of non-specific binding to tissues. The binding of $[^3\text{H}]\text{perhydro-MTX}$ to rat glioma C6 cells was inhibited by a didesulfo-MTX, suggesting the presence of the specific site for MTX-binding on the external surface of the cell membrane.

Molecular Species Analysis of Phospholipids: Phospholipids from pheochromocytoma PC12 cells were purified by thin-layer chromatography. Material corresponding in R_f to phosphatidic acid (PA) was analyzed by fast atom bombardment mass spectrometry (FAB). The molecular ions of the major constituents corresponded in mass to phosphatidylglycerols, which, however, have a lower R_f value. Analysis of the mass spectra demonstrated that this material consists of bis(monoacylglycerol)phosphates (BMP). Linked scans of individual molecular ions indicate that BMP from PC12 cells is esterified almost exclusively with monounsaturated (16:1 and 18:1) and polyunsaturated (20:4 and 22:6) fatty acids. One of the two major molecular species contains two monounsaturated (18:1/18:1), while the other contains both a monounsaturated (18:1) and a polyunsaturated (22:6) fatty acid ester. PC12 cells were stimulated with a phorbol ester or with the calcium-mobilizing receptor ligand bradykinin in media containing 1% ethanol. The fatty acid composition of the molecular species of phosphatidylethanol (PEt), a product of phospholipase D activation, formed in stimulated cells was compared with the molecular species of endogenous phospholipids isolated from unstimulated PC12 cells. PEt was isolated and analyzed by FAB. Fatty acid composition and headgroup structure of the major PEt molecular ions were confirmed by linked scan analysis. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol were isolated from unstimulated cells and converted into phosphatidic acids using phospholipase D. Mass spectra of the respective phosphatidic acids were obtained by FAB. The molecular species of PEt formed in phorbol ester and bradykinin-

stimulated PC12 cell were identical to those of phosphatidylcholine isolated from untreated cells. FAB in combination with thin-layer chromatography is ideally suited for analysis of molecular species of phospholipids.

SECTION ON OXIDATION MECHANISMS

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

In the area of polycyclic aromatic hydrocarbon metabolism and chemistry, synthesis and configurational assignment of four enantiomerically pure, bay-region 10,11-diol 8,9-epoxide diastereomers of dibenz[a,h]acridine from the corresponding optically pure *trans*-10,11-dihydroxy-10,11-dihydrodibenz[a,h]acridine enantiomers has been completed. The present studies utilized short bed/continuously developed preparative TLC plates to separate diastereomeric bis((-)-menthyl oxy) esters of the racemic *trans*-dihydrodiol. Assignment of (10R,11R)-absolute configuration to (-)-*trans*-10,11-dihydroxy-10,11-dihydrodibenz[a,h]acridine was achieved through the application of the exciton chirality circular dichroism technique to the bis[p-(dimethylamino)-cinnamoyl] ester of its tetrahydro analog.

Metabolism of the (+)-(*S,S*)- and (-)-(*R,R*)-1,2-dihydrodiols of triphenylene by rat liver microsomes and 11 purified isozymes of cytochrome P450 in a reconstituted monooxygenase system has been examined. Both enantiomers were metabolized at comparable rates by each preparation. The distribution of metabolites between phenolic dihydrodiols and bay-region 1,2-diol 3,4-epoxide diastereomers varied substantially with the different systems. Only cytochromes P450c (P450IA1) and P450d (P450IA2) had high catalytic activity. With either enantiomer, both purified cytochrome P450c (P450IA1) and liver microsomes from 3-methylcholanthrene-treated rats formed diol epoxides and phenolic dihydrodiols in approximately equal amounts. Purified cytochrome P450d (P450IA2), however, formed bay-region diol epoxides and phenolic dihydrodiols in an 80:20 ratio. Experiments with antibodies indicated that a large percentage of the metabolism by microsomes from control and phenobarbital-treated rats is catalyzed by cytochrome P450p (P450IIIA1).

Bay region diol epoxides react with the purine bases in DNA by alkylation of their exocyclic amino groups. Thus, DNA oligomers of defined sequence with specific diol epoxide adducts are of immense value in studying mechanisms of mutagenesis and carcinogenesis. The bay-region tetrahydrophenanthrene-3,4-epoxide represents a prototype for the carcinogenic bay-region diol epoxides of polycyclic aromatic hydrocarbons. The *trans* N-6 amino adduct of 2'-deoxyadenosine (dA) with the tetrahydroepoxide at the benzylic 4-position was prepared by coupling the *trans* C-4 aminolysis product of the epoxide with the 6-fluoro analog of dA in which the sugar hydroxyl groups were protected as silyl ethers. The resulting pair of diastereomers, after chromatographic separation, were assigned absolute configurations through the use of optically pure epoxide of known configuration. The (3*S*,4*S*)-diastereomer, after appropriate derivatization, has been incorporated in high yield into the deoxypentamer TpGpApGpT to document the utility of the nucleotide coupling as well as the blocking/deblocking procedures in the synthesis of a hydrocarbon adducted oligomer. Synthesis of the *cis* ring-opened epoxide adduct by the exocyclic amino group of 2'-deoxyadenosine (dA) has also been achieved. The approach taken consists of coupling (\pm)-*cis*-3-hydroxy-4-amino-1,2,3,4-tetrahydrophenanthrene

with a 6-fluoro analog of dA in which the furanose hydroxyl groups are protected. The required amino alcohol was obtained by reaction of 1,2-dihydrophenanthrene with osmium tetroxide to form the cis 3,4-diol, conversion to the trans chlorohydrin benzoate via its orthobenzoate, displacement of the benzylic chloride by azide, hydrolysis to the cis azido alcohol, and reduction to the racemic cis amino alcohol. Coupling of the amino alcohol with the 3',5'-bis-tert-butyltrimethylsilyl derivative of 6-fluoro-9-(2'-deoxy- β -D-erythro-pentofuranosyl)purine results in a pair of diastereomers that are readily separated by HPLC on silica gel. Replacement of the previously used pyridine by 2,6-lutidine significantly improved the yield for the coupling step. Both adducts were acetylated on the hydroxyl group of the hydrocarbon and then desilylated on the sugar. Absolute configurations were assigned to the adducts based on the shapes of their CD spectra. The adducts were blocked at the 5'-sugar hydroxyl group with the 4,4'-dimethoxytrityl group and allowed to react with 2-(cyanoethyl)-N,N-diisopropyl chlorophosphoramidite to produce the desired activated nucleosides. Incorporation into the deoxynucleotide TpGpA pGpT as the central base proceeded in good yield with minor modifications to the standard DNA synthesizer protocol.

The synthesis and separation of the diastereomeric trans N²-2'-deoxyguanosine (dG) adducts of tetrahydrophenanthrene 3,4-epoxide and benzo[a]pyrene 7,8-diol 9,10-epoxide (benzylic hydroxyl group and epoxide oxygen trans), as well as the incorporation of the former into the pentanucleotide TpApG pApT, have also been described. Thus, our present methodology allows synthesis of DNA oligomers containing either cis or trans opened diol epoxide adducts at the exocyclic amino group of both purine bases.

The pS189 shuttle vector carrying a *supF* target gene was used to compare the mutagenic specificities of the four configurational isomers of benzo[a]phenanthrene 3,4-diol 1,2-epoxide. One of these isomers is the most tumorigenic dihydrodiol epoxide tested to date and another is essentially inactive as a tumorigen. Overall mutagenicities were not correlated with tumorigenicities, but each configurational isomer induced a unique spectrum of mutational hot spots in the *supF* target gene, which monitors primarily point mutations. It is suggested that the demonstrated isomer-specific selectivity for mutation targets within the *supF* gene may be indicative of a similar selectivity for one gene versus another and that such selectivity may be one determinant of relative tumorigenicity.

Mutations in the coding region of the hypoxanthine (guanine) phosphoribosyltransferase (HPRT) gene of Chinese hamster V-79 cells were examined after exposure of the cells to a high cytotoxic dose (0.48 μ M; 35% survival) and a low noncytotoxic dose (0.04 μ M; 100% survival) of the ultimate carcinogen (+)-7R,8S-dihydroxy-9S,10R-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene [(+)-BPDE]. Independent 8-azaguanine-resistant colonies were isolated and cDNAs were prepared by reverse transcription. The coding region of the cDNA of the HPRT gene was amplified by the polymerase chain reaction and sequenced. An examination of the DNA base sequence changes induced by different doses of (+)-BPDE demonstrated that the high dose of (+)-BPDE caused base substitution mutations almost exclusively at G-C base pairs whereas the low dose of (+)-BPDE caused mutations at both G-C and A-T base pairs. Thus, use of a low dose of (+)-BPDE allowed the detection of mutations (at A-T base pairs) that were not readily observed with a high dose of (+)-BPDE. The data also suggest that the low dose of (+)-BPDE may have caused a different profile of base substitutions at G-C base pairs and exon

deletions than the high dose. The results indicate dosedependent differences in the profile of mutations for an ultimate carcinogen.

SECTION ON OXIDATION MECHANISMS

Mechanistic Enzymology of HIV Proteins

In infectious virions, the active form of the RT of HIV-1 is a heterodimer consisting of subunits of 66 and 51 kDa (p66 and p51, respectively). The amino terminal portion of the p66 polypeptide contains the polymerase domain and is separated from the carboxyl terminal portion, which contains the RNase-H domain, by a central region. In a previous report, we identified two highly conserved amino-acid repeat motifs within this central region: the first resembles a "leucine zipper" motif consisting of four leucines and one threonine, and the other is an unusual tryptophan repeat motif. In the isolate HIV-1_{MO32} the proposed leucine zipper comprises residues 282 through 310 of RT. In an attempt to assess whether this motif behaves analogously to a classical leucine zipper, which should form an α -helix capable of dimerization, a 30 amino acid peptide corresponding to the proposed zipper sequence in this isolate was examined by CD measurements in the far-UV region. In aqueous buffer (pH 7.0) at 25 °C the CD spectrum is characteristic of a random coil conformation, whereas in the presence of 90% trifluoroethanol, an α -helix promoting agent, the CD spectrum exhibits changes indicative of partial (ca. 16 %) α -helix formation. On the basis of this observation as well as molecular dynamics calculations by Carson et al. (NIH/DCRT) we had proposed that interactions between these sequences in the p51 and p66 subunits might be involved in heterodimer formation. Within the past year, the X-ray structure of HIV-1 RT with a bound inhibitor has been solved by Kohlstaedt, Wang, Friedman, Rice and Steitz [Science 236, 1783-1790 (1992)]. In this crystalline heterodimer, the polypeptide-chain regions of the p51 and p66 subunits that comprise our putative leucine zipper-like sequence are not in contact. Thus, interaction between these sequences of the two subunits is not possible, and their function is still unknown.

A continuous microspectrophotometric method was used to measure the rates of hydrolysis of three synthetic peptide substrates catalyzed by the retroviral proteases of HIV-1 and of avian myeloblastosis virus (AMV) as well as the mammalian aspartic acid protease pepsin. The kinetic parameter k_{cat}/K_m for these reactions is markedly increased by increasing the sodium chloride concentration. In all cases, we observed a monotonic increase in rate with increasing salt concentration up to 5.0 M NaCl. This result is inconsistent with several reports in the literature of a bell-shaped dependence of the rate on sodium chloride concentration. These previous studies employed point assays in which partial insolubility of the substrates at high ionic strength may have been undetected. If this were the case, a decrease in apparent rate would be observed at high salt concentrations, because the concentration of substrate available to the enzyme would depend upon its solubility rather than the nominal amount of substrate in the reaction mixture, and bell-shaped curves would result. The use of chromogenic substrates, whose solubilities could be evaluated spectrophotometrically at each salt concentration, eliminated this potential source of error. Detailed kinetic analysis of the hydrolysis of the synthetic peptide, Thr-Phe-Gln-Ala-Phe(NO₂)-Pro-Leu-Arg-Glu-Ala, catalyzed by AMV protease and by pepsin at concentrations of sodium chloride between 2 and 5 M showed that k_{cat} remained constant, whereas K_m for both enzymes and K_i for the inhibition by pepstatin A

of the AMV protease-catalyzed hydrolysis were markedly decreased. Slopes of $\log K_m$ and $\log K_i$ for the AMV protease vs. sodium chloride concentration were identical. The observation that salt concentration does not affect k_{cat} suggests that this salt effect is not due to changes in the secondary structure or extent of dimerization of the retroviral proteases. The similarity of the sodium chloride effects on k_{cat}/K_m for the monomeric enzyme pepsin and for the dimeric retroviral proteases is also not consistent with a salt effect on the monomer-dimer equilibrium of the retroviral proteases. The most likely mechanism for this effect on both K_m and K_i involves a "salting out" phenomenon in which hydrophobic interactions between the substrate or inhibitor and the enzymes' active sites are enhanced at high salt concentrations.

We are continuing collaborative studies with Dr. John Louis [Project No. z01 DK 15509-01 LCDB] on the autoprocessing of the HIV-1 protease. The availability of protease constructs containing flanking Pol region sequences, expressed as fusion proteins with the maltose-binding protein (MBP) of the *malE* gene of *Escherichia coli*, has made possible kinetic studies of their autoprocessing to give enzymatically active protease. The denatured (5 M urea) fusion protein, MBP- Δ Pol-PR- Δ Pol', undergoes renaturation and autoprocessing upon dilution with aqueous buffers in a stepwise process whose time dependence may be followed by i) spectrophotometric assays of protease activity and ii) separation by SDS-PAGE of the protein species formed, and quantitation of the resulting bands by densitometry. Four protein species were monitored during the activation process: the full-length fusion protein, the MBP, a 13.2 kDa intermediate which presumably contains the C-terminal Δ Pol' sequence, and the 11 kDa protease. The latter two species were detected by immunoblotting. The quality and reproducibility of quantitative data derived from the gels were such as to provide highly reliable kinetic results. The appearance of enzymatic activity occurs with a rate constant identical to that for the disappearance of the full-length fusion protein, the appearance of the maltose binding protein, and the appearance of the sum of the 13.2- and 11-kDa species. This process has a half-life of ca. 20 min, and is first-order in protein concentration, consistent with a mechanism in which the fusion protein exists at equilibrium predominantly as a dimeric species, which undergoes intramolecular cleavage of the MBP- Δ Pol fragment in the initial proteolytic step. Conversion of the 13.2-kDa protein to the 11-kDa protease is much slower than the formation of this intermediate, such that its concentration exhibits a typical rise and decay pattern. Since the appearance of enzymatic activity is concomitant with the formation of the 13.2-kDa protein, this species must possess catalytic activity similar to that of the mature 11-kDa protease.

SECTION ON OXIDATION MECHANISMS

Mass Spectrometry of Drugs, Natural Products, Proteins and Oligonucleotides.

Biologically Active Molecules from Plants and marine Organisms: The collaboration on the structural investigation of biologically active natural products from plants and marine organisms has continued. The compounds investigated are those that show promising antiviral, antibacterial and, especially, anti-AIDS potencies, or are synthetic derivatives of these compounds. Compounds with high anti-AIDS activities have been identified.

Calcium Channel Blocker: Effort has been expended to establish the identity of a natural calcium channel blocker and its final identity is almost

confirmed. A compound has been identified and work is continuing to establish its structure.

Anticonvulsant plasma level studies: Three blood studies have so far been completed on anticonvulsant drugs of interest for possible patient clinical trials in the Epilepsy Research Branch of NINCDS. Of particular interest has been the very marked difference in the activity of 1-(3-fluorophenyl)-cyclohexylamine between rats and mice. By an examination of the difference in metabolic functions in these two species, it is possible a more potent anticonvulsant drug may be formulated.

SECTION ON PHARMACODYNAMICS

Adenosine Receptor Agonists and Antagonists

Structure-Activity Relationships for 2-Substituted Adenosines. 2-Alkyloxy-, 2-aryloxy- and 2-aralkyloxy-adenosines were screened as inhibitors of the binding of [³H]R-phenylisopropyladenosine to A₁-adenosine receptors in rat cerebral cortical membranes, and of the binding of [³H]N-ethylcarboxamidoadenosine (NECA) to A_{1A}-adenosine receptors in rat striatal membranes and as agonists at A_{2A}-adenosine receptors coupled to adenylate cyclase in rat pheochromocytoma PC12 cell membranes. The activities are consonant with a hydrophobic binding site in the A_{2A}-receptors at a distance from the 2-position of the adenine ring corresponding to a spacer chain of -O-CH₂-CH₂-. There is little lateral steric tolerance in the region occupied by the spacer chain. The affinities of the 2-substituted adenosines for the rat cerebral cortical A₁ receptors are not as markedly altered by structural changes and in almost all cases are 2- to 100-fold less than the affinity of the 2-substituted adenosine for the rat striatal A₂ receptor. There is an excellent correspondence of the present data on rat A_{2A} receptors with the reported potencies of these 2-substituted adenosines as coronary vasodilators in guinea pig heart preparations.

Adenosine Receptors and Hunting Magic. A frog used for "hunting magic" by Amazonian Indians is identified as *Phyllomedusa bicolor*. This frog's skin secretions, introduced to the body through fresh burns, are rich in peptides. These include vasoactive peptides, opioid peptides, and a new peptide--adenoregulin with the following sequence: G-L-W-S-K-I-K-E-V-G-K-E-A-A-K-A-A-K-A-A-G-K-A-A-L-G-A-V-S-E-A-V. Adenoregulin enhances binding of agonists to A₁-adenosine receptors; it is accompanied by peptides that inhibit binding. Synthetic adenoregulin although identical in molecular weight to natural adenoregulin differs slightly in chromatographic properties, suggesting that the natural peptide contains one or more D-amino acid residues. Synthetic adenoregulin causes a 1.6-fold stimulation of binding of agonists to A₁ receptors. It has no effect on binding of antagonists and in the presence of guanyl nucleotides it is inhibitory to binding. The vasoactive peptide sauvagine, the opioid peptides, and adenoregulin and related peptides affect behavior in mice and presumably contribute to the behavioral sequelae observed in humans.

Chronic Caffeine and Locomotor Activity in Mice. Chronic ingestion of caffeine by mice caused a marked reduction in locomotor exploratory activity. At least four days of withdrawal were required to restore activity to normal levels. Stimulatory effects of injected caffeine were lower in chronically-

treated mice and the biphasic dose-response (stimulatory followed by depressant) curve for injected caffeine was left-shifted. Seven days of withdrawal were required before the dose-response curve to caffeine was identical to that of control mice. The depressant effects of a potent xanthine phosphodiesterase inhibitor, 1,3-dipropyl-7-methylxanthine, were blunted in caffeine-treated mice. The depressant effects of A_1 - and A_2 -selective adenosine analogs were enhanced after chronic caffeine. There was little or no effect of chronic caffeine on the stimulatory effects of dopaminergic agents (amphetamine, cocaine), while both depressant and stimulatory effects of cholinergic agents (nicotine, oxotremorine, scopolamine) were reduced. The results indicate that chronic caffeine affects functions of adenosine receptors and cholinergic receptors that are related to regulation of locomotor exploratory activity.

SECTION ON PHARMACODYNAMICS

Interaction Between Second Messengers

Transfection of G_{q11} in NIH 3T3 cells: coupling to prostaglandin receptors.

Prostaglandin F_2 stimulates phosphoinositide breakdown in NIH 3T3 fibroblasts. Such stimulation seems to be mediated by one or more G proteins. The possibility that the alpha subunits from the G_{q11} subfamily of heterotrimeric G proteins might be capable of coupling PGF_2 receptors to phospholipase C was explored. Such pertussis toxin resistant G protein α subunits have been previously shown to stimulate phospholipase C- β in vitro. NIH 3T3 cells transfected with G_{q11} generated larger amounts of inositol phosphates in response to GTPYS or GTPYS + PGF_2 than in control cells or cells transfected with vector without insert. In transfected cells, the concentration of PGF_2 required to induce calcium mobilization was 100 times lower than the minimal effective concentration to induce calcium mobilization than in control or vector transfected cells. These results suggest that G_{q11} can transduce activating signals to phospholipase C in NIH 3T3 cells and that a PGF_2 receptor is capable of stimulating phospholipase C through this G_q subunit.

Muscarinic receptor-mediated tyrosine phosphorylation of phospholipase C- γ .

In CHO cells transfected with m5 muscarinic receptors, carbachol elicits a marked stimulation of phosphoinositide breakdown and a sustained elevation of $[Ca^{2+}]_i$, the latter dependent on the presence of extracellular calcium. The marine toxin maitotoxin (MTX) elicits similar responses. Carbachol- and MTX-induced sustained elevations of $[Ca^{2+}]_i$ can be inhibited by the proposed blockers of receptor-operated calcium channels, CAI (L651582) and SKF 96365. Both, carbachol and MTX induce a significant increase in total protein tyrosine phosphorylation, as determined by immunoprecipitation of lysates of cells labelled with [32 P]orthophosphate. The increase in tyrosine phosphorylation is dependent on extracellular calcium and can be inhibited by CAI and SKF 96365. Among the substrates subject to tyrosine phosphorylation after carbachol or MTX, phospholipase C- γ (PLC γ) was identified by combination of immunoprecipitation with an antiphosphotyrosine antibody and subsequent immunoblot with an antiphospholipase C- γ antibody. Carbachol-induced [3 H]inositol phosphate formation in CHO-m5 cells is partially inhibited by CAI and is reduced in the absence of extracellular calcium, suggesting that phosphorylation of PLC γ plays a role in the muscarinic activation of phosphoinositide breakdown. Such an effect

of carbachol is reminiscent of effects observed with growth factors and represent a novel alternative signaling pathway for a muscarinic G protein coupled receptor.

Prostaglandin F2 α -mediated stimulation of phospholipase D in NIH 3T3 cells.

Using a transphosphatidylation assay, we have found that PGF₂ stimulates phospholipase D (PLD) activity in NIH 3T3 cells. Prostaglandin E₂ (PGE₂) was inactive on PLD activity at the concentrations tested. PGF₂ also stimulates phospholipase C (PLC) in these cells, but the concentration of PGF₂ required to attain maximal PLD stimulation was 10 times lower than that required for maximal PLC activation. Staurosporin, an inhibitor of protein kinase C, blocks phorbol ester-induced, but not PGF₂-induced PLD activation. The marine toxin maitotoxin (MTX) induces a strong stimulation of PLC, but is virtually inactive on PLD activity in NIH 3T3 cells. These results suggest that PGF₂ activates PLD activity through a mechanism independent from PLC activation. Such mechanism could involve a receptor coupled to PLD via guanine nucleotide binding protein.

Non-Voltage dependent L-Type Ca²⁺ Channel Operated by PGE₁ in NIH 3T3 Cells.

In NIH 3T3 cells prostaglandins can activate phospholipase C activity by interacting with a receptor coupled to a G protein. Such stimulation of phospholipase C by PGs has the following order of potencies PGF₂ > PGD₂ > PGE₂ > PGI = PGE₁. PGE₁ is inactive in stimulating PLC activity in NIH 3T3 cells. PGF₂, PGE₂ and PGD₂ induced mobilization of [Ca²⁺]_i with the same order of potencies shown for PLC stimulation. Surprisingly, PGE₁ was equally efficacious in inducing [Ca²⁺]_i mobilization. PGF₂-induced [Ca²⁺]_i mobilization showed two components, an early response at 15 sec, which was not affected by removal of extracellular calcium, and a late response at 42 sec, that was eliminated in the absence of extracellular calcium. In contrast, PGE₁-induced [Ca²⁺]_i mobilization had a single component, a peak at 6 sec, and it was completely eliminated in the absence of extracellular calcium. PGE₁-induced, but not PGF₂-induced, increase in [Ca²⁺]_i was blocked by a mixture of inorganic calcium channel blockers (0.1 mM CoCl₂, 0.1 mM NiCl₂ and 0.1 mM CdCl₂) and by the L-type calcium channel blockers nifedipine (0.1 μ M) and D-600 (1 μ M). BAY K 8644 (1 μ M), a dihydropyridine that activates L-type calcium channels also induced an increase in [Ca²⁺]_i in these cells. High concentrations of K⁺ induced no change in [Ca²⁺]_i. Although PGE₁ elicited the accumulation of cAMP in NIH 3T3 cells, other agents that elevate cAMP, like forskolin or the phosphodiesterase inhibitor IBMX did not affect [Ca²⁺]_i. Furthermore, PGE₁-induced cAMP was observed even in the absence of extracellular calcium. The results presented strongly suggest the presence of a novel L-type-like calcium channel in NIH 3T3 cells that can be activated by PGE₁. Such activation is not a consequence of the generation of the second messengers cAMP, or the products of phospholipase C stimulation.

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

SECTION ON BIOCHEMICAL MECHANISMS

IMIDAZOLE-BASED ANTIMALARIAL AGENTS

Plasmodium falciparum, one of the four malaria parasites which infect man, developed resistance to chloroquine and other standard antimalarials about 30 years ago in Thailand and Columbia. As the result of its uncontrolled spread, 300 million people are now believed to be infected worldwide and 2 million die every year in Africa alone. To date, neither a drug nor vaccine has been found to combat this plague.

The organism has the unusual property of inducing production, within an invaded erythrocyte, of a protein containing 30-70% histidine. The protein is found in dense knobs on the outer surface of the erythrocyte membrane. The high histidine content would create centers of very high positive charge; thus, the knobs may be responsible for a very strong adherence of the infected erythrocyte to capillary endothelium, thereby sequestering parasitized cells which would normally be destroyed during passage through the spleen. We had initially chosen 2-fluorohistidine for testing because we had previously demonstrated that 2-FHIS can partially replace histidine in de novo protein in both E. coli and mammals. Furthermore, the fluorine atom reduces the pK of the imidazole ring from its normal value of 6 to 1.5; thus, its incorporation into knobs should dilute the clusters of positive charge and reduce cytoadherence. In cultures of infected erythrocytes, low concentrations of 2-FHIS not only do inhibit cytoadherence but also prevent maturation of the parasite and the appearance of knobs entirely. The assumption that these strong antiparasitic effects are due to the incorporation of 2-FHIS into the histidine-rich protein proved unwarranted, since the treated parasite shows a rather low incorporation of labeled 2-FHIS but a general decrease in protein synthesis. As one of several hypotheses for the mechanism of action, we propose that 2-FHIS interferes with histidine on the outer cell surface as a promoter of the transport of other essential amino acids (especially isoleucine) into the cell. This hypothesis is supported by our earlier findings that 2-FHIS inhibits protein synthesis in cell and organ cultures but not in cell-free systems. Unfortunately, the high antimalarial activity shown by 2-FHIS in vitro could not be extended because the compound proved lethal to owl monkeys, even at doses 1/20 that of the LD₅₀ for mice (250 mg/kg).

We then screened a large number of other ring-substituted histidines previously unknown, but only the 2-iodo analog (2-IHIS) showed activity comparable to that of 2-FHIS. Neither 4-X-histidines nor 2,4-di-X-histidines show activity, while 2-iodohistamine is somewhat active; surprisingly, the 2-chloro and 2-bromo analogs are inactive. Thus, the role of the iodine atom cannot be electronic but may be just the right size to plug an erythrocyte membrane channel involved in active or passive transport of a nutrient essential to the parasite. In contrast to 2-FHIS, 2-IHIS does not significantly retard protein synthesis while retarding maturation, a result supporting this mechanism of action. Although 2-IHIS proved nontoxic to monkeys, it retarded growth of parasite for only 24 h. We suspected that the compound may be a substrate for a mammalian deiodinase but, in the course of a search for such a deiodinase, found that the iodine is rapidly removed nonenzymatically under physiological conditions by any sulfhydryl compound (cysteine, glutathione, etc.) present in tissue. This loss of activity would not be observed in vitro because of the low levels of sulfhydryl compounds present in the culture dish. The finding that the deiodination need not be enzyme-mediated greatly reduces the possibility of stabilizing the drug by derivatization of the side chain, by use of the D-histidine series, or by introduction of a bulky alkyl group. Our hypothesis for the mechanism predicts that N-alkylation will not prevent or even retard deiodination, and experimental results support this prediction. Deiodination of thyroxine and its model o-iodophenols is also achieved nonenzymatically with mercaptans (thyroxine deiodinases contain SH or SeH in the active site), but the mechanism differs from that of the iodohistidine case since alkyl-

ation of the phenolic group prevents deiodination.

The mechanism of deiodination requires prior protonation of the imidazole ring; thus, 2,4-diiodohistidine is deiodinated at 1/10 the rate of 2-iodohistidine, because the second halogen reduces ring basicity to a significant degree. Unfortunately, the second iodine also introduces extra bulk and this steric baggage may be the reason the diiodo amino acid is biologically inactive. But the same reduction in basicity can be achieved with much smaller groups, and this fact provides a lead for new analogs. 4-Trifluoromethylhistidine has been iodinated at C-2, and the trifluoromethyl group converted to cyano by exposure to aqueous ammonia. The cyano group is even more electronegative than iodine and very small. While histidine and its protected derivatives undergo bromination and iodination with extreme facility, chlorination has always proved almost impossible to achieve. We have found that "carbonylcyclohistidine", the bicyclic urea obtained by reaction of histidine ester with carbonyldiimidazole, undergoes chlorination very easily to produce both the mono and dichloro derivatives. Iodination of the monochloro derivative, followed by acid hydrolysis, provides the desired 4-chloro-2-iodo-L-histidine. As expected, 2-iodohistidines containing strong electronegative groups at C-4 do not undergo rapid deiodination by mercaptans.

Examination of space-filling models reveals that 2-iodohistidine has a width corresponding exactly to the diameter of the erythrocyte membrane channel, as estimated from diffusion rates of small molecules. The same dimension can be found in metabolically stable molecules, such as 1-isopropyl- and 2-isopropyl-histidine. A program was initiated to develop general synthetic methods for the previously inaccessible ring-alkylated histidines. Several novel approaches have proved successful, and extensive series of 1-alkyl and 2-alkylhistidines are being prepared for screening.

LIGAND-ACTIVATED AFFINITY LABELS (LAAL'S)

The three-dimensional structure of a protein imposes considerable perturbation on various functional groups in its component amino acids. Van der Waals forces, coulombic and electrostatic interactions, hydrogen bonding and other phenomena serve to alter the nucleophilic reactivities of Lewis bases and to perturb the pK values of ionizable groups in both directions. Thus, it is known that various enzymes contain amino, carboxyl, sulfhydryl and phenolic groups with pK values 1-4 units different from those of the same groups in the denatured protein or in the individual amino acids. Such information is available for a number of enzymes, but has not yet been demonstrated for receptor proteins; since abnormal pK is a consequence of the three-dimensional protein structure, similar perturbations should also exist in receptor proteins.

The concept of the suicide substrate has proved very effective as an approach to selective inhibition of enzymes. An analog of the natural substrate, which lacks significant chemical reactivity, serves as a pseudosubstrate and is transformed by the enzyme into a highly reactive entity now capable of forming an irreversible covalent bond to the protein. Since a receptor was not designed to serve as an enzyme, it is not capable of transforming a chemically unreactive ligand or pseudoligand into a reactive one. But receptor protein will have functional groups in the binding site with abnormal pK, and these groups have the potential of donating or removing a proton from a ligand or pseudoligand. The first phase of this project, therefore, was to invent or discover functional groups which are essentially unreactive, at physiological pH, toward the nucleophiles found in body fluids; these same groups, however, must become highly reactive as the result of a proton transfer at some pH modestly different from 7.4. If such a functional group is incorporated into a receptor ligand and selective binding capacity can be retained, proton transfer should activate the ligand and lead to irreversible blocking of the receptor site. This process would not only

assist in identification and isolation of the receptor, but may also have far-reaching clinical implications. Our studies with analogs of peptide hormones have shown that introduction of such functional groups results not only in retention of binding capacity but in an increase in selectivity for multiple receptor systems.

The first functional groups found to meet these requirements were 2-fluoro- and 2-nitroimidazoles; however, the increase in chemical reactivity resulting from gain of a proton proved insufficient for rapid attachment to protein. As a result of studies on mechanisms of activation and electronic substituent effects, we have learned which modifications are necessary to achieve adequate reactivity and the necessary synthetic procedures are being devised. We have used similar analyses of mechanism and substituent effect to enhance (or moderate) the reactivities of the cyano, trifluoromethyl, perfluoroalkyl and orthoester functions. Efforts in progress involve the incorporation of such groups into peptide and nonpeptide hormones, therapeutic drugs, neurotransmitters and nucleosides. Especially critical is the development of routes to radiolabelled materials so that binding studies can be initiated.

CHEMISTRY AND BIOLOGY OF NOVEL PYRIMIDINE AND PURINE NUCLEOSIDES

Despite the countless number of modifications of pyrimidines and purines which have been explored in the search for clinically effective and acceptable anticancer and antiviral agents, very few have achieved the conditions required for safe testing in humans. We have undertaken approaches not yet considered by others, in that the unique physical and chemical properties of the fluorine atom are used in key roles.

Uracil condenses specifically with trifluoroacetaldehyde and hexafluoroacetone at C-5 to form derivatives containing perfluoro alcohols at this critical position. Uracil also reacts specifically with bis(perfluoroalkanoxy)peroxides under radical conditions to give perfluoroalkyl derivatives at the same position. The latter compounds lose two fluorine atoms readily at pH 8-9 to form perfluoroalkyl ketones, which exist preferentially as their hydrates or tetrahedral nucleophilic adducts. Since the half-life for loss of fluorine is ca. 20 min. at pH 8, these compounds are potential irreversible affinity labels for pyrimidine recognition sites *in vivo*. Under the same reaction conditions, the perfluoroalkylpyrimidines are 3-5 times as reactive as trifluoromethylpyrimidines, whose strong antiviral properties have been thoroughly demonstrated. The same radical perfluoroalkylation can be achieved with uridines. Although the carbon-fluorine bond is significantly more stable in the nucleoside series, conversion to ketones can also be achieved in basic media, particularly with sulphhydryl catalysis. These ketones are reducible to secondary perfluoro alcohols with sodium borohydride. The inductive effects of multiple fluorine atoms renders the alcohols highly acidic and readily capable of forming strong internal hydrogen bonds with a carbonyl or amino group at C-4. We anticipate that such hydrogen bonding will interfere with the intermolecular hydrogen bonding needed for effective base pairing in polynuclear strands and, thus, expect that such compounds may block cell division and may act as antiviral and anticancer agents. Furthermore, hydrogen bonding to this position in purines and pyrimidines has been found essential for the action of PRPP synthetase. By increasing the length of the perfluoroalkyl chain, lipophilicity can be correspondingly increased to meet the needs for effective transport across membranes. The nucleophile at C-4 in uridine or cytidine is also capable of displacing a fluorine atom adjacent to the ketone to form novel bicyclic pyrimidine nucleosides. All of these compounds also have the potential of acting as inhibitors of cytidine deaminase. Biological evaluations of the compounds are in progress.

Analogous perfluoroalkyl groups have been introduced into purines, primarily at C-8; these compounds provide an additional series of "preaffinity labels". Our earlier studies on the chemistry of perfluoroalkylimidazoles indicated that such a group at C-8 of a purine should undergo loss of fluorine under physiological conditions and provide, in vivo, a very reactive functional group capable of bonding to a protein or nucleic acid nucleophile. Current efforts involve the attachment of dideoxyribose moieties to these purine analogues.

A major portion of the chemistry involved in this project has been performed under the GIRIN-NIDDK Joint Research Program, sponsored and supported by the Ministry of Industry and Technology of Japan, but with planning and direction provided through the Section on Biochemical Mechanisms, LBC.

BIOINDOLES AND OXINDOLES AS MEDICINAL AND DIAGNOSTIC AGENTS

Radioiodinated melatonin (N-acetyl-5-methoxytryptamine) has been in use for some years as a tool in radioimmune assay. The iodination by ICl occurs at C-2 in very poor yield and produces a variety of other products, necessitating elaborate purification by HPLC. Since we had previously achieved the syntheses of 2-chloro- and 2-bromotryptophan in very good yield, we undertook a reexamination of the iodination problem. Initial efforts to achieve iodination of N-acetyltryptophan methyl ester were very disappointing. Far better results were obtained with the N-trifluoroacetyl derivative (65%) and further study showed that the introduction of all three halogens could be readily achieved without the need for the radical generators used in our earlier work.

This dependence on the nature of the acyl protecting group on the side chain proved quite surprising, and our surprise was reinforced by the finding that the t-BOC protecting group also produced low yields. It appeared that effective halogenation depends on the degree of acidity of the acylamino NH group, with trifluoroacetyl amino being the most acidic of the series. Thus, we adopted the hypothesis that positive halogen first converts α -NH to α -NX and that X then transfers intramolecularly to add to the 2,3-double bond of the pyrrole ring. The resulting halonium species then collapses to an intermediate with halogen at C-2 and a carbocation at C-3, and the pyrrole is regenerated by $E1$ elimination of a proton at C-2. This hypothesis, however, failed to explain the fact that 3-methylindole undergoes facile halogenation at C-2 under the same conditions, without the benefit of intramolecular transfer. Thus, it appeared that direct formation of the halonium intermediate could occur with 3-methylindole but not with the tryptophan derivative. This difference can be rationalized by the realization that the halonium intermediate, in the case of 3-methylindole, collapses quickly to the 2-halocarocation because the methyl group at C-3 is very effective in stabilizing a + charge by hyperconjugation. On the other hand, the amino acid side chain contains electronegative ester and acylamino functions, and is significantly weaker than methyl in stabilizing a carbocation. As a result, the iodonium intermediate of tryptophan tends to dissociate back to starting materials in preference to forming the iodoindole. Only by efficient intramolecular transfer can deiodination be retarded sufficiently to lead to product. Indeed, the N-halogenated species could be detected on TLC plates as an unstable intermediate. Finally, iodination of the N-phthaloyl derivative, in which a side-chain NH is totally lacking, gave a very poor yield of iodo product, showing that direct iodination of the pyrrole can occur without assistance but with considerable difficulty.

It now became obvious that replacement of the N-acetyl group of melatonin by N-trifluoroacetyl should improve the iodination yield considerably, and this alternative is now being explored. To our surprise, the N-trifluoroacetyl analog had never been prepared and its biological activity, therefore, is not known. The compound will soon be evaluated for comparison in activity to the

natural N-acetyl derivative. N-trifluoroacetylserotonin is also being prepared for parallel studies.

Several years ago, we made a concerted effort to obtain 2-fluoroindoles by halogen exchange with 2-bromoindoles, but were unsuccessful. Direct fluorination by use of new fluorinating agents have now been surprisingly successful and such novel compounds are anticipated to find application as affinity labels, analogs of peptide hormones and PET scanning reagents.

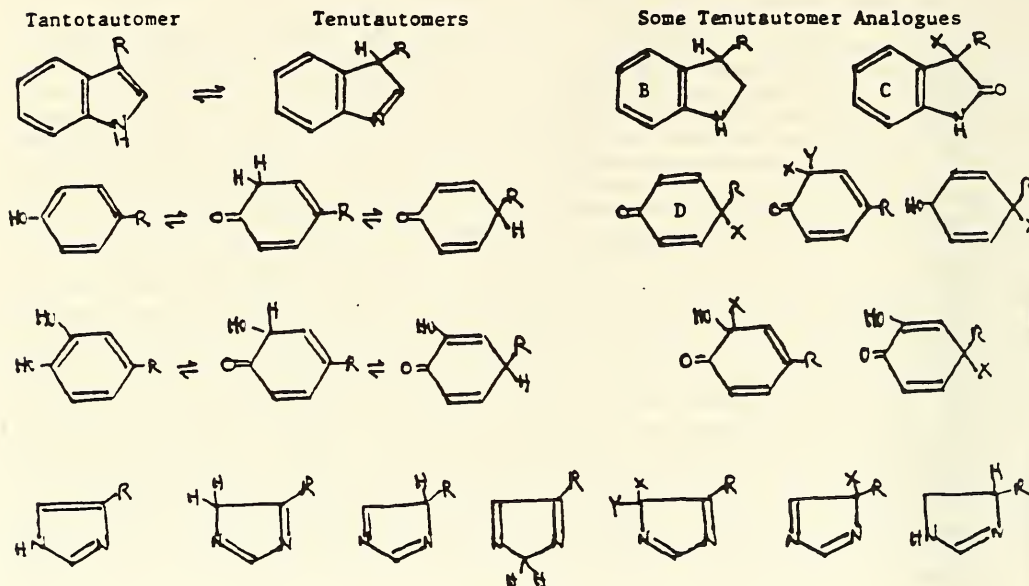
Efforts to retard and ameliorate late-onset diabetic complications have been directed toward reversible and/or irreversible inhibition of the enzyme, aldose reductase. These complications affect the eye (cataracts), kidney, nervous system and circulation; they are thought to result from the hyperosmotic effects of abnormally high intracellular concentrations of sorbitol, in turn resulting from the reduction of excess glucose symptomatic of diabetes. Aldose reductase is the enzyme responsible for sorbitol formation.

Our studies on the synthesis of inhibitors of tryptophan-metabolizing enzymes have produced oxindole derivatives which are somewhat analogous in overall geometry to certain commercial compounds which had reached advanced clinical trials as aldose reductase inhibitors. In vitro assays have revealed that, while these oxindole derivatives (lactones) are not significantly active, their hydrolysis products are as potent as any others thus far developed. Although these chiral compounds are not amino acids, we have succeeded in using chymotrypsin to achieve their resolution by selective ester hydrolysis. Assignment of stereochemistry has been achieved through x-ray crystallography and through the novel approach of destroying the known chiral center while retaining the unassigned center in a diastereoisomer. The introduction of certain substituents on the benzene ring of the oxindole has been found to increase inhibitor activity several fold. Additional analogues have been prepared which have been found to serve as irreversible affinity and photoaffinity labels for the enzyme. Current efforts are being devoted to improvements in yield, in resolution and in enhancement in lipophilicity, with the goal of achieving more effective penetration and transport to the sites of action in vivo. Still another new direction involves the replacement of oxygen by sulfur in the oxindole portion, in order to encourage the formation of a trigonal carbon at C-3. Thus, we hope to learn more about structure-activity requirements from these analogues. Since our inhibitor series is significantly different in structure and in functional groups from those which had reached clinical trials, we have strong hope that this series will not elicit the serious side effects which ultimately had caused many of the commercial candidates to be abandoned.

SIGNIFICANCE OF LIGAND TAUTOMERISM IN BIORECOGNITION

A large variety of metabolically important molecules exist as pairs of tautomers, in which the major tautomer (tantotautomer) is so favored energetically over the minor tautomer (tenu-tautomer) that the latter species cannot even be detected in the equilibrium mixture by any available spectroscopic means. Nevertheless, mechanistic studies have revealed that the tenu-tautomer is very often the "true" reactant in various chemical transformations. We have proposed that the same phenomenon may exist in biological systems, namely, that the tenu-tautomers of various metabolites (Scheme I) may be the "true" ligands for some enzymes and receptors. This theory provides a new pathway for substrate activation; it also leads to the critical consequence that potential inhibitors and antagonists should be designed as analogues of the tenu-tautomers, rather than the common practice of mimicking the tantotautomers. Since it is still impossible to examine the detailed structure of a substrate or ligand within a binding site, arguments for the tenu-tautomer hypothesis must be based on evidence and inference from the behavior of stable analogues of the tenu-tautomer.

This approach was highly successful and convincing in the case of tryptophan. We found that stable analogues of the indolenine tautomer of L-tryptophan with a tetrahedral carbon at C-3—e.g., 2,3-dihydro-L-tryptophan (B), oxindolyl-L-alanine (C, X = H) and dioxindolyl-L-alanine (C, X = OH)—are potent competitive inhibitors of tryptophan synthase and tryptophanase, enzymes involved in the biosynthesis and degradation of tryptophan. Furthermore, the two enzymes show "mirror-image" specificity, in that the 3R diastereoisomer of each analogue inhibits only tryptophanase while the 3S diastereoisomer inhibits only tryptophan synthase. The diastereoisomers of 3-azido-oxindolyl-L-alanine have also been prepared as potential photoaffinity labels for these enzymes. A logical extension of this series involves the synthesis of tetrahedral analogues of the 5-hydroxy and 5-methoxy derivatives of bioindoles, currently in progress. Additional analogues are being designed and synthesized as potential affinity labels for other bioindole enzymes and receptors.



Some years ago, we discovered a synthetic route to the p-hydroxydienone analog of tyrosine by electrolytic oxidation (D, X = OH). Unfortunately, this compound is rather unstable and we have been exploring routes to more stable systems (D, X = OMe, CN, N₃, OAc, alkyl, etc). Several of these stable dienones have now been synthesized and will soon be tested as inhibitors of tyrosine phenol-lyase. Even more important tests and applications of the principle involve the synthesis and evaluation of dienone analogues of catecholamines and dihydroxyphenylalanine; the synthesis of such analogues presents a novel and unprecedented challenge. Studies have also been initiated to explore the synthesis of CH-tautomers of histidine and histamine, for which we have already established a role in test-tube reactions and which we believe to be the true substrates in certain enzymatic processes. Recent results with histidine-ammonia lyase confirm our proposal for the involvement of a histidine CH-tautomer as the "true" substrate for this enzyme.

FLUORINATED ANALOGUES OF BIOACTIVE PEPTIDES

Fluorinated analogues of a wide range of structurally different classes of organic compounds have been prepared and many of these have proven to be important pharmacological and medicinal agents. The advantages of fluorine substitution arise from the small van der Waals radius of fluorine (1.35 Å). As a result, fluorine bonded to a trigonal carbon is used effectively as a C-H replacement. Furthermore, because of similar aliphatic C-F and C-O bond lengths (1.39 and 1.43 Å, respectively) and because fluorine has some ability to participate in hydrogen bonding, the halogen bonded to a tetrahedral carbon has been used frequently as an OH surrogate, particularly in carbohydrate research and in nucleoside analogues. These steric similarities allow fluorinated compounds to mimic their nonfluorinated parents with respect to recognition by various biological macromolecular systems such as enzymes, transport proteins and receptors. On the other hand, the high electronegativity of fluorine (4.0) can drastically alter electron density distribution in the molecule which, in turn, affects pK values in neighboring functional groups and molecular dipole moments. All these altered physicochemical properties of the molecule, as a result of fluorine substitution, can result in drastically modified biological properties such as potency, transport and receptor selectivity. Furthermore, recent advances in 19-F NMR and electron energy-loss spectroscopic techniques, and 18-F positron-emission tomography (18-F PET), have increased the significance and application of fluorinated molecules as biological markers and diagnostic agents.

Although many methods have been developed for fast and efficient incorporation of 18-F and 19-F into various compounds of medicinal importance, such methods have not been generally practical for incorporation into peptides. We have now developed a method for fast, efficient introduction of fluorine regioselectively into the phenolic ring of tyrosine-containing peptides by use of the electrophilic fluorinating agent, acetyl hypofluorite (AcOF). We have used this method for fluorinating μ -selective opioid peptides. In vitro bioassays and receptor-binding assays have revealed that these fluoro peptides retain their high receptor selectivity. Thus, we have been able to develop a very μ -selective fluoro peptide [Tyr(3-F)-D-Arg-Phe-Lys-NH₂], which is already in use as a research tool to probe the complex opioid receptor system. This unique method of fluorination also permits the facile incorporation of radioactive fluorine into peptides and other biological materials.

By use of a different fluorinating agent, N-fluoropyridinium triflate, fluorination at the C-2 position of tryptophan has been achieved. Extension of this method to tryptophan-containing peptides is in progress. Studies are under way to determine whether different fluorinating agents, by virtue of differences in reaction mechanism, can achieve selective fluorination of different amino acids.

NOVEL AMINO ACIDS FOR CONFORMATIONAL AND STEREOCHEMICAL CONSTRAINTS IN PEPTIDES

Introduction of conformational constraints in peptides is a very useful means to probe peptide-receptor interactions and to enhance their potencies and/or selectivity. While local conformational restraints can be imposed by incorporation of unsaturated or small ring amino acids, side chain-side chain cyclization to form cyclic analogues has been found to be one of the most useful approaches for the introduction of more general conformational constraints: e.g., a disulfide bond between Cys or Pen residues, or an amide bond between side chains of amino and carboxy trifunctional amino acids. The 13-membered cyclic peptide, H-Tyr-D-Orn-Phe-Asp-NH₂ (cyc-Orn-Asp), is a potent μ -selective opioid peptide. Molecular modeling of this peptide has revealed considerable flexibility in the ring structure, making it difficult to identify a single conformation which may be biologically relevant. In order to restrict further the conformational mobility in such peptides, we have designed and synthesized a novel trifunctional amino acid based on pyrrolidine (PDA). This amino acid, if used in place of Orn or Lys for side-chain-side chain cyclization, is expected to reduce considerably the conformational flexibility

in the macrocyclic ring of these peptides owing to the introduction of a bicyclic structure. Moreover, the additional asymmetric center in the pyrrolidine ring may introduce differential stereochemical constraints to the binding of diastereoisomeric peptides, derived from the corresponding diastereoisomeric PDA's. Thus, these peptides may act as probes for delineating the stereochemical topology of the receptor in the vicinity of the ring structure and may have useful biological properties and applications. Synthesis in the 2-pyrrolidinealanine series had been completed last year and parallel efforts in the 3-pyrrolidinealanine series are almost finished.

Optically active amino acids are being used increasingly as synthons for the preparation of a variety of chiral compounds. PDA's can be expected to be excellent synthons for compounds such as pyrrolizidines with the stereochemistry already defined at two centers. Furthermore, these amino acids may possess antibacterial or other antimetabolic activities. An efficient method for the synthesis of all four optically active stereoisomers of 2-PDA has been developed. These amino acids will be used for the preparation of novel bicyclic opioid peptide analogues.

ANALOGUES OF THYROTROPIN-RELEASING HORMONE

In addition to governing the release of thyrotropin and prolactin from the pituitary, TRH (L-pyrroglutamyl-L-histidinyl-L-prolineamide) is known to exert a wide variety of effects on both the central nervous system (CNS) and the cardiovascular system (CVS). TRH has shown promise in the treatment of circulatory shock and/or CNS ischemic damage, as a promoter of the regeneration of injured spinal cord, as an antidepressant and in the amelioration of symptoms associated with amyotrophic lateral sclerosis (ALS). However, the great variety of its biological effects presents a serious drawback to its use as a drug for specific purposes. Our early studies with synthetic analogues of TRH (involving modification of the imidazole ring of histidine) had suggested that some of the central actions of TRH are not mediated through the recognized high affinity TRH receptors and that appropriate analogues may achieve the long sought specificities of action.

Receptor binding studies with some of our new analogues have made it clear that endocrine and various centrally mediated actions of TRH involve uniquely different mechanisms and that, after several decades of effort in various laboratories, the separation of these activities has been achieved. Thus, 4(5)-NO₂-Im-TRH is highly selective for CVS activity, and may be useful in the treatment of various forms of shock without any endocrine effects. On the other hand, Nva²-TRH is a selective analeptic agent without effect on the cardiovascular system; this analogue has served as a research tool for delineation of those binding sites in rat brain which may mediate the analeptic effects of TRH and its analogues. Computer-assisted structure-activity analysis of a large number of imidazole-substituted analogues has helped us design more selective and potent analogues, as well as photoaffinity labels for TRH receptors. Thus, our predictions of the vital role of imidazole-NH tautomer preference in determining selectivity in binding by brain receptors has been validated by the demonstration that 1-Me-5-NO₂-Im-TRH binds ca. 3000 times as tightly as 1-Me-4-NO₂-Im-TRH. Recently, we have carried out receptor-binding analysis of various TRH analogues with subtle backbone modifications (replacement of the peptide amide with a thioamide surrogate); these studies have allowed us to identify subtle differences in the high affinity TRH receptors in rat pituitary and brain. Current efforts are devoted to the synthesis and evaluation of analogues of TRH designed to meet the requirements of our new concept of "receptor-activated affinity labels" (RAAL's). Ideally, such a compound would block a receptor site selectively and indefinitely under conditions of clinical use.

Analogues of TRH in which histidine has been replaced by 2-pyrroleanine or by 3-pyrroleanine have now been synthesized. These analogues are expected to confirm our hypothesis that the multiple TRH brain receptors fall into two classes, each binding a different tautomer of TRH. Thus, we expect that the pyrrole peptides, as stable analogues of the respective TRH tautomers, will each exhibit one or more of the separate activities of TRH.

Two TRH analogues—4-NO₂-Im-TRH and 2,4-I₂-Im-TRH—have been found to improve behavioral recovery following traumatic brain injury in rats. Because 4-NO₂-Im-TRH has little endocrine activity and 2,4-I₂-Im-TRH has minimal cardiovascular effect, these results support the hypothesis that the neuroprotective actions of TRH and its analogues are independent of their endocrine or autonomic activities.

Analogues of TRH have been prepared which contain thioamide moieties in the pyroglutamic acid ring, the carboxamide proline terminus, and in both positions. The monothioamide analogues are comparable to TRH in their ability to release TSH in vitro or in vivo. The second analogue shows a higher affinity for pituitary receptors than TRH itself, while the former analogue had lower affinity and reduced selectivity. Thus, the subtle exchange of sulfur for oxygen can have a significant impact on both receptor selectivity and affinity for a bioactive peptide.

CHEMISTRY OF IMIDAZOLES AND BIOIMIDAZOLES

Perfluoroalkylimidazoles have attracted considerable interest because of their utility in providing lipophilic sites in medicinal agents and because of their potential use as "receptor-activated affinity labels" (RAAL's) for irreversible blocking of receptors in chemotherapy. The simplest and most general methods for perfluoroalkylation of all heterocyclic rings have been developed in this laboratory and are based on the photochemical generation of perfluoroalkyl radicals. Photochemical perfluoroalkylation of imidazoles leads to mixtures of 2- and 4-isomers while the reaction with pyrroles occurs only at C-2. The practicality of the method for complex substrates has been demonstrated by the facile trifluoromethylation of histidine in peptides (e.g., TRH), followed by EPLC separation of the isomers. Exposure of the trifluoromethyl derivatives of TRH to aqueous ammonia leads to their conversion to the corresponding cyano derivatives. All of these derivatives show very selective binding to low affinity TRH receptor sites in the brain. We have now developed a procedure for regiospecific substitution by blocking all undesired sites with the methylthio group, which is subsequently removed with Raney nickel. Thus, 3-perfluoroalkylpyrroles have been made for the first time. Perfluoroalkylimidazoles have been found to undergo facile nitration and halogenation without destruction; therefore, methods are now available to prepare photosensitive ligands (azido, diazo) and ligands labeled with radioiodine. We have also synthesized pentafluoroethylhistidine and histamine isomers by photochemical radical substitution. These compounds are converted by base into the corresponding trifluoroacetyl derivatives, which have such reactive carbonyl groups that they may serve as affinity labels for bioimidazole-binding sites. Upon treatment with methanolic base, the trifluoromethyl analogs can be converted into trimethoxymethyl and the pentafluoroethyl derivatives into ketals. These ortho functionalities are also of interest as potential covalent affinity labels.

Over the past 16 years, we have found consistently that 2-X-bioimidazoles (especially X = fluoro and iodo) have a broad range of strong biological activities but the corresponding 4-X-bioimidazoles are essentially inactive. We now believe

that 2-X-bioimidazoles are recognized and bound to receptors because they have the same NH-tautomer preference as natural bioimidazoles, while the 4-X series prefer the unnatural tautomer. While 4-X-bioimidazoles are readily accessible by direct electrophilic substitution, 2-X-bioimidazoles can be obtained only by indirect and, often, very tortuous routes. Prior to our major efforts in this area, the majority of 2-X-bioimidazoles were totally unknown. The numerous methods available for simple imidazoles are not applicable to bioimidazoles, because the additional functional groups cannot be adequately protected and because chirality should be preserved. Thus, nonclassical methods (e.g., photoradical substitution, electrochemistry, B-12 catalysis, cobalt oxygen carriers, one-electron reduction, singlet oxygen oxidation) have been developed to fit these gaps. The past year has seen the discovery of three major new synthetic methods: (1) the totally specific electrophilic dehalogenation of 2,4-dihaloimidazoles to give 2-haloimidazoles, thus making 2-iodohistidine very easily available for antimalarial studies; (2) the use of transient ylid-carbenes to provide a route to 2-X-imidazoles not otherwise accessible; (3) the discovery that 2-aminoimidazoles are readily oxidized to 2-nitroimidazoles by singlet oxygen, thus providing inexpensive routes to new radiosensitizers and selective cytotoxic agents for cancer chemotherapy.

We have now demonstrated that 2-nitroimidazoles are far more chemically reactive than supposed, and that loss of the nitro group follows both ionic and radical pathways. These results provide the first support for our theory that 2-nitroimidazoles act as radiosensitizers and cytotoxic agents by affinity alkylation with imidazole radicals, and not by the generally assumed reduction of the nitro group to the hydroxylamine stage.

Our studies on the chemical reactions of cyclic urea derivatives of histidine and histamine have opened entirely new directions in achieving regiospecific substitution in bioimidazoles. Parallel studies are under way with cyclic sulfones of these imidazoles and appear likely to provide extremely useful protection for histidine in peptide synthesis.

STEREOPOPULATION CONTROL IN DRUG DELIVERY AND ENZYME SIMULATION

Enzymes accelerate organic chemical reactions by 10-20 powers of ten over their closest test-tube counterparts. For example, at pH 7 and ambient temperature, the half time for conversion of adenosine to inosine by adenosine deaminase is 1.9 nsec; in the absence of enzyme, the same reaction would require nearly 15,000 years! In order to account for this remarkable catalytic power of enzymes, it is usually considered that the activation free energy is contributed both by binding of the substrate to the enzyme (step 1) and by chemical transformation of the bound substrate (bond-making and breaking, step 2). Popular opinion holds that most of the activation energy is supplied in step 2. We have proposed, however, that the overall catalytic process is more easily justified on the assumption that the first step contributes a more significant share of the activation energy than is generally accepted. To support this theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen into a single, very favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (stereopopulation control). These compounds undergo intramolecular reactions (independently of any functional assistance) at rates comparable to those catalyzed by enzymes, and show that the protein raises both the entropic and enthalpic components of the substrate by binding it in a single, rigid conformation. Recent work has involved a study of steric and electronic effects on NMR and IR spectra across tight space rather than through covalent bonds. These studies show that kinetic and spectral properties are linearly related to the van der Waals size of crowding substituents. The upper limit of energy-related steric crowding

could not be evaluated because of inability to introduce the very bulky iodo group. After considerable effort, this goal has been reached. The thermal Balz-Schiemann method for introduction of the fluorine atom at C-5 failed with the 6-mesylate, but the same reaction on the unprotected phenol gave a 29% yield of the desired fluorodihydrocoumarin. Direct fluorination has also been achieved by use of N-fluoro-3,5-dichloropyridinium triflate, although in lower yield. Efforts are continuing to introduce the trifluoromethyl group, the last of the bulky substituents planned for the present series.

Studies with the series of substituted o-nitrophenylpropionic acids as potential "Pro-Prodrugs" have made significant headway with the completion of the parent derivatizing groups. To date, they have been coupled to benzylamine, GABA methyl ester, and a protected DOPA derivative. Preliminary kinetic investigations have shown that chemical reduction of the nitro group in both series occurs easily and essentially at the same rate, while cyclization to the lactam (with release of the group on the acyl carbon) is indeed hastened by the presence of the gem-dimethyl group on the alpha carbon.

SECTION ON DRUG RECEPTOR INTERACTIONS

Functionalized Congeners of Bioactive Compounds - By the functionalized congener approach to drug design, new analogs are synthesized with the regiospecific inclusion of a functionalized chain at a point which can accommodate molecular modification and a certain degree of steric bulk. The resulting functionalized drug congener may then be attached through an amine or other reactive group on the chain to various organic moieties, such as amines and peptides. The receptor-binding affinity of these analogs is often greater than that of the parent drug and does not necessarily diminish as the molecular weight is systematically increased. XAC (xanthine amine congener), a high affinity adenosine antagonist, was designed by this approach.

Applications of the functionalized congener approach include the synthesis of probes for receptor studies (for example: radioligands, fluorescent probes, or chemically reactive affinity labels) and the design of drug delivery systems (including targeting and altering the characteristics to allow for passage through membranes). Drug conjugates may be designed in a stepwise approach to optimize certain pharmacological properties, such as potency, specificity, and duration of action.

Functionalized congeners are also being explored for therapeutic goals. Most clinically available cholinergic drugs (e.g. the agonist pilocarpine and the antagonist atropine) are non-selective in their interaction with muscarinic receptor subtypes. The recent cloning, sequencing, and expression of five separate genes for muscarinic receptors has raised the possibility of developing novel organic compounds that act as agonists or antagonists at one of these subtypes. Selective compounds could be therapeutically useful in treating a variety of diseases, including Alzheimer's disease, cardiac disease, neurogenic bladder, and certain sleep disorders. Furthermore, such specific compounds, by virtue of their subtype selectivity, should be devoid of many of the side effects of currently used compounds.

In view of the above, a goal is to develop novel and selective muscarinic antagonists. We have used a functionalized congener approach in the design of derivatives of pirenzepine and telenzepine. The attachment of a spacer chain to the distal piperazinyl nitrogen was based on previous findings of enhanced affinity at muscarinic receptors in an analogous series of alkylamino derivatives of pirenzepine. The potency/selectivity were highly dependent on the number of methylene groups. The most potent derivatives contained a 10-aminodecyl group, which provided a nucleophilic functionality for further derivatization. The amines were acylated with various reporter groups resulting in molecular probes of nanomolar affinity. The telenzepine derivatives contain prosthetic groups for radioiodination, protein cross-linking, photoaffinity labeling, fluorescent labeling, and biotin for avidin complexation. We explored the effect of chain length on aryl isothiocyanate derivatives that were found to be receptor affinity labels. The affinity for muscarinic receptors in rat forebrain (mainly m1 subtype) was determined in competitive binding assays vs. [^3H]N-methylscopolamine. A p-amino-phenylacetyl derivative for photoaffinity labeling had a K_i value of 0.29 nM at forebrain muscarinic receptors (16-fold higher affinity than telenzepine). A biotin conjugate displayed a K_i value of 0.60 nM at m2-receptors and a 5-fold selectivity versus forebrain. The high affinity of these derivatives make them suitable for the characterization of muscarinic receptors in pharmacological and spectroscopic studies, for peptide mapping, and for histochemical studies. [Kenneth Jacobson]

Prosthetic Groups for Labeling of Functionalized Drugs and Peptides - Binding sites for certain drugs in an animal or organ may be localized as a result of the synthesis of

high specific activity radiolabelled analogs which have high affinity for that binding site. Prosthetic groups may be attached to a drug or other receptor ligand for the purpose of efficient and selective chemical capture of a particular radioisotope. Having developed functionalized congeners of theophylline and other drugs acting at adenosine receptors, we are now developing prosthetic groups for radioisotopes such as ^{18}F , ^{123}I , and ^{125}I , to be coupled to these functionalized drug molecules. The prosthetic groups contain amino or carboxylic groups which are to be condensed covalently to functionalized drugs to give conjugates of high affinity at a particular receptor. These prosthetic groups contain amino or carboxylic groups which are to be coupled covalently to functionalized drugs to give conjugates of high affinity at a particular receptor.

Positron emission tomography (PET) based on short-lived isotopes such as ^{18}F and ^{11}C has been used for imaging receptors in the brain and other organs. A prosthetic group for chemical capture of ^{18}F requires rapid and efficient reaction and purification, since the half-life is only 110 minutes. Compounds, such as fluorodeoxyglucose, containing this isotope have been used extensively as an *in vivo* tracers. ^{18}F positron emission tomography (PET) has been particularly effective in the study of brain metabolism and in scanning receptors and sites of drug uptake. PET scanning allows more than spatial imaging; it provides a look at the biochemical state of an organ.

An objective of our chemical investigation is to provide compounds and a general methodology for rapid introduction of radioactive fluorine into peptides and functionalized drugs. This general methodology for introduction of ^{18}F is hoped to lead to improvements in diagnostic nuclear medicine, and to provide improved brain imaging by positron emission tomography.

The reactions through which ^{18}F is introduced into a molecule include nucleophilic displacement of alkyl triflates, aromatic nucleophilic attack, and halogenation of phenols, olefins, and metallo-organics. Benzylic electrophilic centers are subject to attack by active nucleophiles, including "naked" fluoride ion in non-aqueous media.

The feasibility of attachment of the bromomethylbenzoyl prosthetic group in biological systems has been demonstrated. In addition to the many peptides which contain non-essential amino groups, some drug molecules may also be coupled to this prosthetic group without loss of receptor binding.

^{18}F -Insulin of high specific radioactivity was synthesized using a new, general methodology for the incorporation of a positron emitter into peptides and proteins. ^{18}F -Insulin is chemically stable and retains the essential biological properties of insulin, as measured *in vitro* by receptor binding, by stimulation of glucose metabolism in rat adipocytes, and in localization studies *in vivo* in primates. The synthesis uses 4-bromomethylbenzoyl- ("BMB") as an amide-linked prosthetic group. Nucleophilic displacement of the bromide by cyclotron-produced ^{18}F -fluoride ion forms a 4-fluoromethylbenzamide. A site-specific reaction at the amino site that is least sensitive in receptor binding (the B^1 phenylalanine residue) was achieved. Specific activities of 12-14 $\text{Ci}/\mu\text{mole}$ at the time of injection typically were obtained. ^{18}F -Insulin that had undergone radioactive decay competed for ^{125}I -insulin binding to insulin receptors in a manner indistinguishable from unlabeled insulin. Unlabeled insulin competed with ^{18}F -insulin in a fashion similar to its competition for ^{125}I -insulin, providing further support for the close resemblance of the fluoro- derivative to the iodo- as well as the native peptide. In *in vivo* studies, Rhesus monkeys were injected with 0.3-1.4 mCi of ^{18}F -insulin and scanned for

30-60 min. Significant uptake of the radiotracer was detected immediately post-injection in liver and kidneys, with delayed uptake in bile and bladder. In the presence of coinjected, unlabeled insulin labeling of the liver decreased by 50%, indicating specific binding of the radiotracer to insulin receptors.

A prosthetic group methodology was utilized to achieve both radioiodination and receptor affinity labeling. Functionalized congeners derived from XAC (an amine congener of 1,3-dipropyl-8-phenylxanthine), a potent A_1 selective adenosine antagonist, were derivatized to contain chemically reactive groups capable of reaction with nucleophiles such as amines and thiols on biopolymers. A new radioiodinated xanthine (adenosine antagonist) that binds covalently to A_1 -adenosine receptors was prepared via a general "trifunctional approach" to receptor ligands and used as a receptor probe. BH-DITC-XAC was synthesized via reaction of XAC with a trifunctional aryl diisothiocyanate crosslinker, containing the p-hydroxyphenylpropionyl group ("BH", Bolton-Hunter reagent) for radioiodination. ^{125}I -BH-DITC-XAC, prepared directly by the chloramine T method and purified by HPLC, bound specifically to A_1 receptors. This binding was inhibited in the presence of the adenosine agonists *R*-PIA, *S*-PIA, and NECA in a dose dependent manner and with the order of potency characteristic of bovine A_1 receptors. Incubation of affinity purified bovine A_1 -receptors (prepared using a XAC-Sepharose affinity column) with ^{125}I -BH-DITC-XAC (0.8 nM) for 2 hours resulted in the specific and clean labeling of a polypeptide band corresponding to MW 36,000, identical to that previously found for the A_1 receptor. [Kenneth Jacobson]

Development of Drugs Acting at Adenosine Receptors - Adenosine acts as a neuromodulator in the circulatory, endocrine, immune and central nervous system. The biological activity is associated with two receptor subtypes: the A_1 -adenosine receptor mediates cardiac and central depressant and antilipolytic activities and is coupled to adenylate cyclase in an inhibitory manner; the A_2 -receptor is involved in vasodilation and antithrombotic functions, possibly through stimulation of adenylate cyclase. The major class of antagonists, the alkylxanthines, generally acts at both receptor subtypes; there is currently a search for analogs which have high potency, high receptor subtype selectivity, and increased water solubility.

Adenosine acts as a neuromodulator through at least two receptor subtypes, A_1 and A_2 . A_2 receptors have been further divided into A_{2a} (high agonist affinity, in striatum) and A_{2b} (low agonist affinity, in fibroblasts) receptors. A number of effector mechanisms (cAMP, PI, ion channels) are activated by adenosine, the best known being adenylate cyclase (inhibited by A_1 and activated by A_2). Several developments within the past few years have enabled a rigorous examination of the molecular structure and regulation of adenosine receptors: 1) the synthesis of adenosine derivatives, as agonists, and xanthine and non-xanthine antagonists with receptor subtype selectivity; and 2) the cloning of both A_1 and A_{2a} adenosine receptors. Our goal is to define the parameters of the binding and receptor activation by purines using a variety of means, including synthetic, genetic, spectroscopic, and computational methodology.

We have synthesized new adenosine receptor ligands using a "functionalized congener approach", by which potential positions for attachment of chains on a pharmacophore are empirically probed. The site of attachment must correspond to a region of relaxed steric requirements at or near the receptor binding site. This strategy has allowed us to target accessory sites of favorable interaction on the receptor, and actually enhance the affinity of the ligands. We have used such congeners to make fluorescent probes, super-potent lipid conjugates, affinity labels, etc. An A_1 selective affinity label was used to

demonstrate the presence of spare adenosine receptors in the A-V node of the guinea pig heart. In collaboration with Dr. Gary Stiles of Duke University Medical Center, A_{2a} receptors have been labeled using the iodinated agonist PAPA-APEC (Figure 1), suggesting a molecular weight of 45,000 Daltons (bovine striatum). The measured molecular weights of the receptor proteins are in good agreement with the values calculated from sequences. The rabbit (striatum), rat (PC12 cells), and frog (erythrocyte) A_{2a}-receptors are of molecular weight 44-47K, and hamster (DDT₁-MF2 cells) A_{2a}-receptors appear to be 40K. In membranes containing the rabbit A_{2a} receptors, cleavage of a 7000 MW fragment was observed in the absence of inhibitors of enzymatic proteolysis. This segment most likely corresponds to the long C-terminal tail found in the A_{2a} but not in the A₁ receptor. The receptor 38K fragment still binds ligands appropriately, however the ability of guanine nucleotides to modulate the coupling to G-protein is enhanced following cleavage. Desensitization of both A₁ and A₂ adenosine receptors in DDT₁ MF-2 smooth muscle cells was observed.

We have characterized the human A_{2a} receptor through radioligand binding and photoaffinity labeling. A truly selective A_{2a} antagonist radioligand is lacking, however one may use [³H]XAC (8-[4-[[[(2-aminoethyl)amino]carbonyl]methyl]-oxy]phenyl]-1,3-dipropylxanthine) for certain species, such as primates and rabbit in which the A_{2a} receptors have particularly high affinity for 8-phenyl xanthines. Although A₁-selective in the rat brain, [³H]XAC binds to human striatal A_{2a} receptors with high affinity (K_d = 2.98 nM). 25 nM CPX (8-cyclopentyl-1,3-dipropylxanthine), an A₁-selective antagonist, in the incubation medium effectively eliminated 91% of [³H]XAC (1 nM) binding to human A₁ receptors, yet preserved 90% of binding to A₂ receptors. [³H]XAC exhibited saturable, specific binding to A_{2a} sites with a K_d of 2.98 nM and a B_{max} of 0.71 pmol/mg protein (25°C, non-specific binding defined with 0.1 mM NECA). The potency order for antagonists against 1 nM [³H]XAC was CGS15943A > XAC = PD115,199 > PAPA-XAC > CPX > HTQZ = XCC = CP-66,713 > theophylline = caffeine, indicative of A_{2a} receptors. A_{2a}-adenosine receptors were detected in the human cortex, albeit at a much lower density (~5%) than in the striatum. Photoaffinity labeling using ¹²⁵I-PAPA-APEC revealed a molecular weight of 45K, with 43K and 37K proteolytic fragments also observed. In the absence of proteolytic inhibitors, the 37K fragment, which still bound ¹²⁵I-PAPA-APEC, was predominant.

Isothiocyanate derivatives of adenosine have been developed as selective affinity labels for A₁- and A₂-adenosine receptors [Jacobson et al, 1992b]. An amine functionalized congener, APEC (2-[(2-aminoethylamino)carbonyl]ethylphenyl-ethylamino]-5'-N-ethylcarboxamido-adenosine), is a selective agonist at A_{2a}-adenosine receptors. As a means of covalently inhibiting this receptor binding site, APEC was coupled to *m*- and *p*-phenylene-diisothiocyanate (DITC). The resulting isothiocyanate derivatives in preincubation with rabbit or bovine striatal membranes irreversibly inhibited radioligand binding at A_{2a}- but not A₁-receptors. Inhibition was prevented by theophylline (antagonist) or NECA (agonist). *p*-DITC-APEC (100 nM) diminished the B_{max} for [³H]CGS 21680 binding by 71% (K_d unaffected), suggesting a direct modification of the ligand binding site. Selective inhibitors are potentially of interest in studies of the physiological role of adenosine receptors.

Detailed amino acid sequence analyses of A₁- and A_{2a}-adenosine receptors were assembled by analogy to other G-protein coupled receptors. The models have been correlated with pharmacological observations with both native and transiently expressed mutants of bovine A₁ receptors in which either His²⁵¹ or His²⁷⁸ residues have been

substituted with Leu. Sites for phosphorylation, palmitoylation, and sodium binding have been proposed. These models conform with the seven trans-membrane domain topology commonly found for receptors linked to G-proteins. Asparagine residues that are potential glycosylation sites are located on E-II in both the A₁ and A₂ sequences. The A₁ and A₂ sequences display a number of consensus patterns for phosphorylation by PKA, PKC or by casein kinase II (CK2). The carboxyl terminus of the A₂ receptor is rich in serine and threonine, suggestive of phosphorylation by bARK. Many G-protein linked receptors contain a cysteine residue in the C-terminal tail close to H-VII, that is palmitoylated and essential for G-protein. The role of an analogous cysteine residue present in the carboxy tail of the A₁ receptor (Cys³⁰⁹), but not the A₂ receptor, is yet to be determined. The sequence [SN]-L-A-x-[AT]-D occurs near the cytoplasmic end of H-II in both A₁ and A₂ receptors and in many other G-protein linked receptors, where it is important in the allosteric modulation of agonist binding by Na⁺. The inhibition of A₂-radioligand binding by the histidyl-modifying reagent diethylpyrocarbonate suggested the involvement of His residues in interactions with adenosine agonists and antagonists. We have shown that radioligand binding to A₂ receptors is disrupted following treatment with the disulfide reactive reagents mercaptoethanol (>50 mM) or DTT or dithionite (>10 mM). This suggests that disulfide linkages indeed are involved in maintaining the structural integrity of the A₂ receptor.

In collaboration with Dr. Ad IJzerman we have recently devised a preliminary molecular model for the binding of adenosine to the A₁ receptor, that is consistent with the observed pharmacology, SAR, and calculated ligand conformation. This model may be used to predict sites for interaction between specific amino acid residues of the receptor and its ligands, and may be tested in studies of mutagenesis of the receptor. Eventually the objective will be to synthesize improved ligands, including chemical affinity labels and possibly ligands of high selectivity for other subtypes (e.g. A_{2b}), consistent with the model.

5'-Ether derivatives of the potent adenosine agonist N⁶-cyclopentyladenosine (CPA) were designed as "caged" ligands for the activation of A₁-adenosine receptors following *in situ* photolysis. The synthesis involved a 2',3'-diol protection scheme using the acid labile ethoxymethynyl group. Generation of CPA was demonstrated chromatographically and in a bioassay measuring the inhibition of synaptic potentials in the rat hippocampus. The penetration of peripherally administered adenosine antagonists into the brain was determined using an *ex vivo* binding technique (CRADA with Cortex Pharmaceuticals, Inc). [Kenneth Jacobson]

Biological Properties of Fluorinated Neuroactive Amines - Putative interactions of the benzylic OH group and the fluoro substituent have been considered as contributing factors to explain the effects of fluorine substitution on the adrenergic selectivities of adrenergic agonists. We now have investigated fluorinated analogues of three types of agonists that have significantly different juxtapositions of a side-chain OH group and the aromatic fluorine, including the natural phenethanolamine agonists, a phenoxypropanolamine agonist, and analogues wherein the benzyl OH is replaced with the hydroxymethyl group. From the striking reduction of beta-adrenergic potency resulting from fluorine on the 6-position of the aromatic ring of the phenoxypropanolamine on the binding interactions with the beta-adrenergic receptor, we concluded that a direct, conformational bias produced by an intramolecular interaction between the OH group and fluorine does not contribute significantly to the reduced beta-adrenergic activity of 6-fluoro-analogues of these compounds. While we observe the same trend in adrenergic activity resulting from fluorination of the hydroxymethyl analogues, the effects are much less dramatic. The effect is more striking for the beta-2 than for the beta-1 response. For each receptor sub-type, an approximately 2-fold increase in potency is seen with the 2-fluorinated analogue. Such a modest increase is consistent with our previous results with fluorinated epinephrines. A significant 7-fold decrease in potency of the 6-fluoroanalogue was found at the beta-2-adrenergic receptor, while this analogue was equipotent with the parent compound at the beta-1-adrenergic receptor. We note that the parent 3-amino-2-(3,4-dihydroxyphenyl)-1-propanol is less potent than 3-amino-1-(3,4-dihydroxyphenoxy)-2-propanol or the phenethanolamine beta-adrenergic agonists we previously studied and it appears that effects on selectivity may be greater for more potent agonists. For example, fluorine had no apparent effect on the adrenergic activity of dopamine, a compound with significant, albeit weak, adrenergic activity. This lack of sensitivity to fluorine substitution had been an initial strong argument for the importance of the beta-hydroxyl group in determining adrenergic selectivities of fluorinated analogues. We are now examining more closely the potential relationship between adrenergic potency and sensitivity to fluorine substitution.

Several points remain to be addressed. First, we have no direct evidence bearing on the mechanism by which a 2-fluoro-substituent adversely affects alpha-adrenergic activity, although the "anti-symmetric" nature of the fluorine effects have led us to believe that, in both the 2- and 6-fluoro series, similar mechanisms probably are involved. Research underway to address this point includes the synthesis of the fluorinated analogues of a (dihydroxyphenyl)morpholine, a compound possessing both alpha- and beta-adrenergic activity, and which also possesses no benzylic OH group. We are also preparing 2- and 6-fluorinated analogues of the enantiomers of dobutamine. The (-)-isomer of 4 is predominantly an alpha-1 adrenergic agonist while the (+)-isomer is predominantly a beta-1 and beta-2 adrenergic agonist [Kenneth Kirk, Bang-Hua Chen, Jun-Y. Nie]

As a first step in studying the effects of the azido group (a pseudo halogen) on biological activity in this series, we used a tyrosine phenol lyase-catalyzed condensation of pyruvate with 3-azidophenol to prepare 2-azido-L-tyrosine. We have prepared 4-azidocatechol diacetate in order to attempt a similar enzymatic synthesis of 6-azido-DOPA. We have prepared 6-azidodopamine by chemical synthesis from N-trifluoroacetyl-6-nitrodopamine diacetate. [Kenneth Kirk, Daniel Appella, Arthur Crossman, Jr.]

Development of Potential Chemotherapeutic Agents - Pteroyl-L-glutamyl gamma-peptides are the predominant form of intracellular folate coenzymes. Polypoly-glu hydrolase sequentially cleaves gamma-glu peptide bonds to produce tetrahydrofolate, the form required for thymidine biosynthesis. A strategy for new and selective antifolate agents involves inhibition of this enzyme. Our initial strategy involved synthesis of difluoromethylene ketone analogues of the glu-gamma-glu as inhibitors of this enzyme.

However, a recent report described the synthesis of sulfonamide peptide isosteres as potential transition state analogues. We have synthesized a L-glu-gamma-L-glu dipeptide sulfonamide-containing analogue from L-homocystine. Subsequent pteridine coupling will produce the desired inhibitor. [Kenneth Kirk, Fabian A. Jameison]

Development of Multifunctional Chemotherapeutic Agents - Amantadine is a well known antiviral agent which has been commercially available for years under the trade name symmetrel. Its primary use is for the prevention of influenza A infections. It also has been reported that at higher doses, it is active against other viral strains. Its mode of action is thought to be at an early stage in replication, probably at the stage of uncoating. In a rational approach to increasing the effectiveness of amantadine, as well as of other antiviral agents, two molecules of amantadine were connected by a linear bridge containing extremely hydrophilic guanidine residues. Interaction of this connecting bridge with viral cell wall glycoproteins was expected to deliver the amantadine residues to the viral surface, thus greatly increasing the effective concentration of the drug. This and related structures were found to be very active against gram positive and gram negative bacteria, fungi, yeast, and enveloped viruses, including herpes simplex and retroviruses. The analogue incorporating the N-chloro functionality also was extremely potent. The exact mechanism of action of these analogues is unknown. The antiviral effect may be due to interaction with or through the viral envelope. [B. V. Shetty]

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31100-27 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacologically Active Compounds from Amphibians and Other Natural Sources

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and

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Others:	C.R. Creveling	Research Chemist	LBC, NIDDK
	T. Spande	Research Chemist	LBC, NIDDK
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COOPERATING UNITS (If any)

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LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

4.4

PROFESSIONAL:

3.9

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Natural products provide a wide range of biologically active agents, many of which have unique profiles of pharmacological activity and therapeutic potential. Over three hundred alkaloids have been identified in extracts from amphibian skins. These included batrachotoxins, which are potent activators of sodium channels, histrionicotoxins, which are noncompetitive blockers of nicotinic receptor channel complexes and of potassium channels, and pumiliotoxins, which have myotonic and cardiotoxic activity due to inhibitory effects on closing of sodium channels. Further alkaloids included 2,5-disubstituted decahydroquinolines, 5,8-disubstituted indolizidines, 1,4-disubstituted quinolizidines, 3,5-disubstituted pyrrolizidines, pumiliotoxins, homopumiliotoxins and allopumiliotoxins, and tricyclic alkaloids, including pyrrolizidine oximes, cyclopenta[b]quinolizidines, cocaine alkaloids, and the potent nonopioid analgesic epibatidine. Homobatrachotoxin has been discovered in feathers, skin and muscle of a New Guinean bird. Characterization of alkaloids in non-dendrobatid amphibians indicated that the biosynthetic pathways to certain dendrobatid alkaloids have evolved separately in one lineage (genus) of amphibians from the families Bufonidae, Myobatrachidae and Ranidae and in a lineage leading to four genera in the family Dendrobatidae. The lack of alkaloid production in captive-raised dendrobatid frogs remains an enigma. Synthetic routes to epibatidine and to the pyrrolizidine oximes have been developed. There was no correlation between inhibition of batrachotoxin binding and anticonvulsant activity for a series of analogs of carbetapentane.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31101-23LBC

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacology and Metabolism of Biogenic Amines and Related Compounds

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others: J. W. Daly, Chief, LBC, NIDDK

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F. Gusovsky, Senior Staff Fellow, LBC, NIDDK

COOPERATING UNITS (if any)

Brossi, A, LAC, NIDDK; Guroff, GF, NICH; Newman, AH, NIDA, Balt. MD; Grossman, M, U. Penn., Phil.PA; Weisz, J., U. Penn., Hershey, PA; Inoue, H., Matsumoto College, Nagano, Japan; Thakker, D., Glaxo Inc., Res. Triangle, NC; Youdim, MBH, Technion

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TOTAL STAFF YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The biochemistry, physiology, and pharmacology of biogenic amines, amino acid precursors and metabolic products, and various synthetic derivative have been investigated. The general areas of study include the effects of fluorine substitution of the properties of biogenic amines, adrenergic antagonists and amino acids and catechol-O-methyltransferase (COMT). Studies on COMT include: 1) immunohistochemical localization and induction of COMT in the luminal epithelium of pregnant and pseudopregnant rat following treatment with progesterone-receptor inhibitors including RU486 2) localization of COMT in macrophages of the corpus lutea and cervical lymph nodes of rat 3) activity and localization of COMT in the hamster kidney and in estrogen-induced carcinomas of hamster kidney 4) activity and localization of COMT in human endometrial and breast adenocarcinomas 5) substrate specificity and reaction kinetics of 3'-hydroxycocclaurines and the identification of berbines as additional products of the O-methylation reaction derived from 3'-hydroxycocclaurine 6) other studies include the identification of monoamine oxidase A and B as one of the irreversible binding sites of procaine isothiocyanate. Studies of fluorine substituted derivatives of biogenic amines include the tissue distribution and incorporation of 2-fluorohistidine in mouse in vivo.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31102-21 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ion Channels, Receptors and Second Messengers in the Nervous System

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and

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TOTAL STAFF YEARS:

2.3

PROFESSIONAL:

2.0

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Calcium, sodium, potassium, and magnesium ions after translocation through ion channels or by transport proteins can cause activation of release processes, contractile proteins, adenylate and guanylate cyclase, phosphodiesterases, protein kinases, phospholipases, ATPases and other enzymes. Receptors of various types and various toxins serve to modulate ion channels and generation of second messengers including cyclic nucleotides, diacylglycerides, arachidonic acid and phosphatidic acid. Maitotoxin (MTX), a ladder-like polyether of molecular weight 3424, activates calcium uptake and phosphoinositide breakdown in all cells studied to date. Activation of phospholipase C by MTX is dependent on extracellular calcium. A blocker of receptor-mediated calcium entry, SK&F 96365, antagonized in a similar dose-response relationship MTX-elicited calcium influx, MTX-elicited phosphoinositide breakdown and MTX-elicited insulin release in cultured cells. Thus, it appears that the primary effect of MTX is to activate directly the SK&F 96365-sensitive so-called receptor-mediated calcium entry system and, thereby, increase internal calcium, activate phosphoinositide breakdown and trigger release of hormones and neurotransmitters. MTX does not act as an ionophore and stimulates uptake of other cations to a much lesser degree than calcium. Binding data suggests a high affinity membrane site. Thin-layer chromatography in combination with fast-atom bombardment mass spectrometry was shown to be ideally suited for identification and analysis of molecular species of phospholipids. Bis(monoacylglycerol)phosphates were identified as significant membrane phospholipids in pheochromocytoma cells. Phosphatidylcholine was shown to be the major substrate for phorbol ester and bradykinin-activated phospholipase D in such cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31104-24 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. M. Jerina	Section Chief	LBC, NIDDK
Others:	J. M. Sayer	Research Chemist	LBC, NIDDK
	H. Yagi	Visiting Scientist	LBC, NIDDK
	A. M. Cheh	Research Chemist	LBC, NIDDK
	N. T. Nashed	Special Expert	LBC, NIDDK
	M. K. Lakshman	Visiting Fellow	LBC, NIDDK
	B. Zajc	Visiting Fellow	LBC, NIDDK

COOPERATING UNITS (If any)

A. Conney, Rutgers U. (Newark, NJ); W. Levin and A. Wood, Roche Research Center (Nutley, NJ); D. Whalen, Dept. of Chem., Univ. of MD (Catonsville); R. Lehr, Dept. of Chem. Univ. of Oklahoma (Norman, OK); R. Loncharich, DCRT, NIH

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

6.5

PROFESSIONAL:

5.5

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The primary goal has been the elucidation of the structures of reactive metabolites responsible for the carcinogenic, cytotoxic and mutagenic activity of drugs, polycyclic aromatic hydrocarbons, and other environmental chemicals. The approach taken consists of: i) synthesis of primary and secondary oxidative metabolites, ii) study of the metabolism of the chemicals with liver microsomes and with purified cytochromes P450 and epoxide hydrolase, iii) evaluation of the mutagenicity and tumorigenicity of the synthetic metabolites, iv) elucidation of the roles of the cytochrome P450 system and epoxide hydrolase in modulating the mutagenicity of these metabolites, v) determination of the rates and products of reactions of arene oxides and diol epoxides with biopolymers and model compounds, and vi) search for agents capable of preventing the tumorigenicity of reactive metabolites. Most of the effort in the past year has concentrated on DNA adducts of carcinogenic bay-region diol epoxide metabolites. A strategy has been developed for synthesis of oligonucleotides containing N6-2'-deoxyadenosine and N2-2'-deoxyguanosine adducts of diol epoxides where the exocyclic amino groups of the purine bases are bonded to the hydrocarbon by cis or trans opening of the epoxide at the benzylic position. The required N-substituted purines are prepared from hydrocarbon aminotriols and appropriately blocked fluorine analogs of the bases. The blocking groups were selected such that standard phosphoramidite chemistry can be used on an automated DNA synthesizer. Such adducted DNA oligomers are expected to be of immense value in the study of mechanisms of mutagenesis and carcinogenesis. Mutations in the coding region of the hypoxanthine (guanine) phosphoribosyltransferase (HPRT) gene of Chinese hamster V-79 cells were examined with the carcinogen (+)-7R,8S-dihydroxy-9S,10R-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. Although this carcinogen is known for causing point mutations at guanine, studies at very low, noncytotoxic doses have indicated that comparable numbers of mutations also occur at adenine, suggestive that there is a small number of A-T hot spots which are obscured by mutations at G-C base pairs when higher nonenvironmental doses are used.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK31106-05 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanistic Enzymology of HIV Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	D.M. Jerina	Section Chief	LBC, NIDDK
Others:	J.M. Sayer	Research Chemist	LBC, NIDDK
	N.T. Nashed	Special Expert	LBC, NIDDK
	J.E. Tropea	IRTA Fellow	LBC, NIDDK
	J.G. Baillon	Visiting Fellow	LBC, NIDDK

COOPERATING UNITS (If any)

J.M. Louis, LCDB, NIDDK; S.H. Wilson, LB, NCI

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.1

PROFESSIONAL:

3.0

OTHER:

0.1

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Enzymology, kinetics and synthetic, physical and analytical chemistry are being used to investigate the mechanisms of catalysis by and assembly of the reverse transcriptase (RT) and protease of human immunodeficiency virus type 1 (HIV-1), with the ultimate goal of developing specific inhibitors for these enzymes. i) In a previous report, we described an amino-acid repeat motif, resembling a modified "leucine zipper", in the RT of several HIV-1 and HIV-2 isolates, which we suggested might be involved in subunit association. X-ray studies by Kohlstaedt et al. have now indicated that the regions containing this motif in the p51 and p66 subunits are not in contact in the crystalline heterodimer. Thus, the function of this highly conserved sequence is as yet undefined. ii) Studies on the acceleration by sodium chloride of the rate of peptide hydrolysis catalyzed by retroviral proteases as well as by the model mammalian enzyme, pepsin, are now complete. This salt effect, which is almost exclusively on the Michaelis constant, is suggested to result from the enhancement of hydrophobic interactions between the substrate and the enzyme's active site. Using a spectrophotometric assay, which facilitated assessment of substrate solubility, we have observed a monotonic increase in rate with NaCl concentrations up to 5 M. Literature reports of a bell-shaped dependence of the rate on salt concentration presumably result from artifacts due to the failure to detect reduced solubility of the substrate at high salt concentrations. iii) Kinetic studies on the autoprocesing of constructs of the HIV-1 protease containing flanking Pol region sequences and expressed as fusion proteins with the maltose-binding protein of *Escherichia coli* are in progress. Preliminary results suggest an initial loss of the N-terminal sequence containing the maltose-binding protein to give a 13.2-kDa intermediate which retains the C-terminal Pol sequence; this intermediate is cleaved to the 11-kDa protease in a slower step. At the concentrations used, the initial cleavage is first-order in protein concentration, consistent with a rapid and favorable dimerization of the fusion protein followed by intramolecular proteolysis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31107-05 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mass Spectrometry of Drugs, Natural Products, Proteins and Oligonucleotides.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	L.K. Pannell	Visiting Scientist	LBC, NIDDK
Others:	D.M. Jerina	Section Chief	LBC, NIDDK

COOPERATING UNITS (If any)

Becker, Whittaker and White, LAC, NIDDK; Daly, Spande, Garraffo and Holbrook, LBC, NIDDK; Fales and Jones, LBC, NHLBI; Munro and Blunt, U. Cant., N.Z.; Hanbauer, LCP, NHLBI; Rogawski and Yamaguchi, ERB, NINCDS; Boyd, NCI, FCRC; Stein, NIST

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Specialized mass spectrometry analyses are provided to the laboratory and to other collaborating units. The emphasis of this work is split between identification of trace organic compounds isolated from biological sources and the mass spectrometry of biopolymers (e.g. proteins). A very close working relationship is maintained with the Laboratory of Biophysical Chemistry, NHLBI, and, with the move into macromolecule mass spectrometry, has been extended to pursue and develop techniques in this area. A major portion of this projects effort has been devoted to the development of methods for handling, digesting and mass analyzing biopolymers. An electrospray source was attached to the JEOL SX102 instrument, and this is now producing mass spectra of macromolecules. A capillary zone electrophoresis instrument was obtained and is being used to study digestion methods on-line to establish the optimum stopping points for protein cutting methods. This has also been interfaced to the electrospray source in a preliminary fashion where its separation efficiency far surpasses that of traditional HPLC methods. As part of the macromolecule collaboration, LBC, NHLBI, has installed a mass spectrometer especially set up for sequencing of protein digests. In the small molecule area, collaborative interest has continued in the identification of biologically active natural products, especially those of interest in AIDS research and treatment; several plasma drug level studies have been completed in collaboration with NINCDS. Samples analyzed derive from many facilities and researchers.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31108-04 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Adenosine Receptor Agonists and Antagonists

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and

P.I.	J.W. Daly	Chief	LBC, NIDDK
Others:	F. Gusovsky	Senior Staff Fellow	LBC, NIDDK
	O. Nikodijevic	Visiting Associate	LBC, NIDDK
	R. Moni	Visiting Fellow	LBC, NIDDK
	W. Padgett	Biologist	LBC, NIDDK
	Dan Shi	Special Volunteer	LBC, NIDDK

COOPERATING UNITS (if any)

R. Olsson, U. So. Fla., Tampa, FL; C. Mueller, U. Tubingen, Germany.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.8

PROFESSIONAL:

2.3

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Adenosine regulates a wide range of physiological functions through interaction with at least two major classes of adenosine receptors. The A1 class of adenosine receptors is inhibitory to adenylate cyclase, while the A2 class is stimulatory to adenylate cyclase. Subclasses of adenosine receptors also occur. Some of these are inhibitory to calcium channels, some are stimulatory to potassium channels, some activate guanylate cyclase, some modulate phospholipid, turn over and some cause smooth muscle relaxation through a poorly defined mechanism. Activities of a range of 2-substituted adenosines led to development of model for the binding site on A_{2A}-adenosine receptors. This consisted of a hydrophobic site separated from the 2-position of the adenine ring by a region with little lateral tolerance. Affinities at A_{2A}-receptors correlated well with potencies as vasodilators. Peptides inhibitory or stimulatory to binding of agonists were discovered in secretions from an Amazonian hyliid frog. The stimulatory peptide adenoregulin had the following sequence GLWSKIKEVGKEAAKAAKAAAGKAALGAVSEAV. It appears likely that one or more D-amino acid residues are present. Chronic caffeine in NIH strain male white mice results in a reduction in locomotor exploratory activity. The threshold for stimulation of locomotor activity to caffeine is lowered as are the thresholds for depressant effects of adenosine analogs. The depressant effects of nicotine are abolished, while those of a muscarinic agonist, oxotremorine are slightly reduced. Thus, chronic caffeine appears to significantly alter function of central adenosine and cholinergic pathways.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31109-03

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interaction between second messengers

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Fabian Gusovsky	Senior Staff Fellow	LBC, NIDDK
John W. Daly	Chief	LBC, NIDDK
Motohiko Takemura	Visiting Fellow	LBC, NIDDK
John Lueders	Biological Laboratory Technician	LBC, NIDDK
Yangmee Shin	Visiting Fellow	LBC, NIDDK

COOPERATING UNITS (if any)

A. Weissman (EIB, NCI), P. Skolnick (LN, NIDDK), M. Beaven (LCP, NHLBI), C. Felder (LCB, NIMH), P. Blumberg (LCCTP, NCI), V. Manganiello (LCM, NHLBI), C. Collin (NS, NINDS), J. Baumgold (G. Washington University, Washington, D.C.).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS.

3.2

PROFESSIONAL:

2.2

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Interactions between signal transduction pathways involving calcium mobilization, and pathways that involve activation of protein tyrosine kinases were studied. In NIH 3T3 cells, the marine toxin maitotoxin (MTX), and PGF₂ α activate phosphoinositide breakdown and induce tyrosine phosphorylation of several protein substrates as determined by immunoprecipitation of [³²P]orthophosphate-labeled cells with an antiphosphotyrosine antibody. Among the substrates subject to tyrosine phosphorylation after PGF₂ α , phospholipase C- γ (PLC γ) was identified by combination of immunoprecipitation with an antiphosphotyrosine antibody and subsequent immunoblot with an antiphospholipase C- γ antibody. The stimulation of protein tyrosine phosphorylation is dependent on the presence of extracellular calcium and it can be inhibited by the blocker of receptor-operated calcium channels SK&F 96365. PGF₂ α -induced phosphoinositide breakdown is primarily attained through a receptor coupled to a G protein which in turn activates most likely PLC β . However, PGF₂ α -mediated phosphoinositide breakdown is partially inhibited by SK&F96365 and is reduced in the absence of calcium. These results suggest that PLC γ may play a role in PGF₂ α -induced stimulation and are reminiscent of effects observed with growth factors. Stimulation of tyrosine kinase activity may be involved in cell proliferation observed with PGF₂ α . In contrast to PGF₂ α , PGE1 is inactive in stimulating phosphoinositide breakdown in NIH 3T3 cells. However, PGE1 induces elevation of intracellular calcium. Such response is dependent on the presence of extracellular calcium and it can be blocked by L-type channel blockers like nifedipine and methoxyverapamil. PGE1 also stimulates the accumulation of cyclic AMP in these cells. The activation of calcium influx, however was not mimicked by agents that elevate cyclic AMP, like forskolin and IBMX. Therefore, in NIH 3T3 cells a receptor for PGE1 is linked to the opening of an L-type-like calcium channel. This channel is not sensitive to depolarization with high concentrations of K⁺, but it can be activated by the dihydropyridine BAY K 8644. This PGE1-mediated regulation of a calcium channel represents a novel mechanism for PG receptor signalling.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31110-16 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analogues of Thyrotropin-releasing Hormone

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Other:	Catherine Dong	Visiting Fellow	NIDDK-LBC
	Albinus D'Sa	Visiting Fellow (term 1/92)	NIDDK-LBC

COOPERATING UNITS (if any)

A. Spatola, Louisville, KY; C. Stammer, Athens, GA; A. Siren, USUHS; S. Vonnhof, HSUS; I. Paakari, Helsinki, Finland; A. Faden, Washington, DC; Y. Tache, Los Angeles, CA; V. Labrop, Seattle, WA; D. Jakobowitz, NIMH.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.8

PROFESSIONAL:

0.7

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In addition to governing the release of thyrotropin and prolactin from the pituitary, TRH (L-pyroglutamyl-L-histidiny-L-prolineamide) is known to exert a wide variety of effects on both the central nervous system (CNS) and the cardiovascular system (CVS). TRH has shown promise in the treatment of circulatory shock and/or CNS ischemic damage, as a promotor of the regeneration of injured spinal antidepressant and in the amelioration of symptoms associated with amyotrophic lateral sclerosis (ALS). Analogues in which histidine has been replaced by our new amino acids, 2-pyrroleanine and 3-pyrroleanine, have now been synthesized. These analogues are expected to confirm our hypothesis that the multiple TRH brain receptors fall into two classes, each binding a different tautomer of TRH. Thus, we expect that the pyrrole peptides, as stable analogues of the respective TRH tautomers, will each exhibit one or more of the separate activities of TRH.

Two TRH analogues-4-NO₂-Im-TRH and 2,4-I₂-Im-TRH-have been found to improve behavioral recovery following traumatic brain injury in rats. Because 4-NO₂-Im-TRH has little endocrine activity and 2,4-I₂-Im-TRH has minimal cardiovascular effect, these results support the hypothesis that the neuroprotective actions of TRH and its analogues are independent of their endocrine or autonomic activities.

Analogues of TRH have been prepared which contain thioamide moieties in the pyroglutamic acid ring, the carboxamide proline terminus, and in both positions. The monothioamide analogues are comparable to TRH in their ability to release TSH in vivo or in vitro. The second analogue shows a higher affinity for pituitary receptors than TRH itself, while the former analogue had lower affinity and reduced selectivity. Thus, the subtle exchange of sulfur for oxygen can have a significant impact on both receptor selectivity and affinity for a bioactive peptide.

Current efforts are devoted to the synthesis and evaluation of analogues of TRH designed to meet the requirements of our new concept of "ligand-activated affinity labels" (LAAL's). Ideally, such a compound would block a receptor site indefinitely.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31111-22 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Stereopopulation Control in Drug Delivery and Enzyme Simulation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen
Other: Michael M. King

Chief, Section on Biochem. Mech.
Special Volunteer

NIDDK-LBC
GWU

COOPERATING UNITS (if any)

Y. Ueno, Nagoya, Japan; W. Antkowiak, Poznan, Poland; Y. Takeuchi, Toyama, Japan; Y. Kikugawa, Tokyo, Japan, W. Durckheimer, Frankfurt, Germany; M. Impicciatore, Parma, Italy; J. Flippen-Anderson, NRL, Washington, DC.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have synthesized a large variety of test-tube systems which simulate the enzyme-substrate complex by having the substrate frozen into a single, very favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (stereopopulation control). These compounds undergo intramolecular reactions at rates approaching those catalyzed by enzymes (but independently of any functional assistance). Recent work has involved a study of steric and electronic effects on NMR and IR spectra across tight space rather than through covalent bonds. These results simulate the spectral perturbations to be expected in a tight ES complex. The upper limit of energy-related steric crowding could not be evaluated, however, because of inability to introduce the very bulky iodo group. After considerable effort, this goal has been reached and we are now attempting to introduce the trifluoromethyl group, the last of the bulky substituents planned for the present series.

As part of our studies of practical applications of stereopopulation control, we have been exploring the use of various derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate transport from the gut to the circulatory system to the brain by temporary masking of charge within the molecule, by improvement in lipophilicity and by regeneration based simply on local pH variation in receptor sites or on local concentrations of potent reducing agents. Studies with o-nitrophenylpropionic acid as potential "Pro-ProDrugs" have made significant headway with the completion of the parent derivatizing groups. To date, these carriers have been coupled to benzylamine, GABA methyl ester, and a protected DOPA derivative. Preliminary kinetic investigations have shown that chemical reduction of the nitro group occurs easily in both series and at the same rate, while cyclization to the lactam (with release of the group on the acyl carbon) is indeed hastened by the presence of the gem-dimethyl group on the alpha carbon.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31112-16 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemistry of Imidazoles and Bioimidazoles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Other:	Rahul Jain	Visiting Fellow (EOD 12/91)	NIDDK-LBC
	Ellen Goldberg	IRTA Summer Fellow	NIDDK-LBC
	Tuan Tran	Stay-in-School	NIDDK-LBC

COOPERATING UNITS (if any)

H. Kimoto, Nagoya, Japan; M. Nishida, Nagoya, Japan; E. DeClercq, Louvain, Belgium; A. Shanzer, Rehovot, Israel; W. Nagai, Nagoya, Japan; J. Retez, Karlsruhe, Germany; S. Avramovici, Jerusalem, Israel; W. Durckheimer, Frankfurt, Germany.

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Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

0.3

OTHER:

0.7

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The past year has seen the discovery of three major new synthetic methods: (1) The totally specific electrophilic dehalogenation of 2,4-dihaloimidazoles to give 2-haloimidazoles, thus making 2-iodohistidine and 2-iodohistamine readily available for antimalarial studies; (2) the use of transient ylid-carbenes to provide a route to 2-X-imidazoles not otherwise accessible; (3) the development of cyclic urea derivatives of histidine and histamine in opening totally new directions for achieving regiospecific ring substitution in bioimidazoles. Parallel studies are under way with cyclic sulfones of these imidazoles and appear likely to provide extremely useful protection for histidine in peptide synthesis. This method provides the first practical route to mono- and dichlorohistidine.

We have now demonstrated that 2-nitroimidazoles are far more reactive chemically than supposed, and that loss of the nitro group follows both ionic and radical pathways. These results provide the first support for our theory that 2-nitroimidazoles act as radiosensitizers and cytotoxic agents by affinity alkylation with imidazole radicals, and not by the generally assumed reduction of the nitro group to the hydroxylamine stage.

Perfluoroalkylimidazoles have been found to undergo facile nitration and halogenation without destruction; therefore, methods are now available to prepare photosensitive ligands (azido, diazo) and ligands labeled with radioiodine. Perfluoroalkylimidazoles have attracted considerable attention because of their utility in providing lipophilic sites in medicinal agents and because of their potential use as "ligand-activated affinity labels." These compounds are converted by mild base into perfluoroalkyl ketones, which have such reactive carbonyl groups that they may serve as irreversible affinity labels for bioimidazole-binding sites in enzymes and in receptors. Perfluoroalkylimidazoles are prepared by photochemical reaction or by condensation of the imidazole (or mercaptoimidazole) with bis(perfluoroalkanoyl)peroxides.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31113-16 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Halogenated Biogenic Amines in Biochemistry and Pharmacology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I. Kenneth L. Kirk Chief, Section on Drug-Receptor Interactions LBC, NIDDK

Other:

Bang-Hua Chen	Visiting Fellow	LBC, NIDDK
Jun-ying Nie	Visiting Assoc.	LBC, NIDDK
Fabian Jameison	IRTA Fellow	LBC, NIDDK
Arthur Crossman, Jr.	IRTA Fellow	LBC, NIDDK
Daniel Appella	Summer IRTA	LBC, NIDDK
J. W. Daly	Chief	LBC, NIDDK
C.R. Creveling	Research Chemist	LBC, NIDDK

COOPERATING UNITS (if any)

M. Channing, D. Kiesewetter (CC, Dept. of Nuclear Medicine), D.S. Goldstein (HE, NHLBI), Claire Fraser (NIAAA), R.S. Phillips (Univ. of Georgia).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Drug-Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

5.4

PROFESSIONAL

5.0

OTHER

0.4

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided)

Biogenic amines play key roles in neurotransmission, metabolism, and in control of various physiological processes. Using a variety of synthetic methodologies, including novel procedures developed by us, we have prepared a series of biogenic amines with fluorine substituted at various ring-positions. By virtue of its very small size and high electronegativity, fluorine is a very favorable replacement for hydrogen in these analogues. The biological properties and usefulness of these ring-fluorinated biogenic amines have proved to be extremely rewarding and continue to find applications in a multitude of studies, including research on the mechanisms of transport, storage, release, metabolism, and modes of action of these amines. Of particular significance was the discovery that 6-fluoronorepinephrine is a selective α -adrenergic agonist and 2-fluoronorepinephrine is a selective β -adrenergic agonist. Mechanisms considered to explain these results include: 1) a direct effect of the C-F bond on agonist-receptor interaction or 2) an indirect effect of the C-F bond on the conformation of the ethanalamine side-chain. The results of testing of new analogues synthesized to probe these mechanisms indicate that electronic effects may be more important than conformational factors. Fluorinated analogues are useful biological tracers. For example, [^{18}F]-labeled 6-fluorodopamine, the biological precursor to 6-fluoronorepinephrine, has been found to be an excellent scanning agent for peripheral noradrenergic innervation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31114-10 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Significance of Ligand Tautomerism in Biorecognition

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen

Chief, Section on Biochem. Mech.

NIDDK-LBC

Other: Catherine Dong

Visiting Fellow

NIDDK-LBC

COOPERATING UNITS (if any)

Robert Phillips, Athens, GA; H. Kimoto, Nagoya, Japan; J. Flippen-Anderson, NRL, Washington, DC; W. Durckheimer, Frankfurt, Germany; Shelly Avramovivi, Jerusalem, Israel; M. Impicciatore, Parma, Italy.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.3

PROFESSIONAL:

0.3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A large variety of metabolically important molecules exist as pairs of tautomers, in which the major tautomer (Tantotautomer) is so favored energetically over the minor tautomer (Tenutautomer) that the latter species cannot even be detected in the equilibrium mixture by any available spectroscopic means. Nevertheless, mechanistic studies have revealed that the tenutautomer is very often the "true" reactant in various chemical transformations. We have proposed that the same phenomenon may exist in biological systems, namely, that the tenutautomers of various metabolite may be the "true" ligands for some enzymes and receptors. This theory provides a new pathway for substrate activation; it also leads to the critical consequence that potential inhibitors and drugs should be designed as analogues of the tenutautomers, rather than the standard practice of mimicking the tantotautomers. We were very successful in using this approach in designing inhibitors of tryptophan synthase and tryptophanase, and are now applying the principle to indole-2,3-dioxygenase.

Some years ago, we discovered a synthetic route to the p-hydroxydienone analogue of tyrosine by electrolytic oxidation. Unfortunately, this compound is rather unstable, and we have been exploring routes to more stable analogues of the dienone tautomer of tyrosine. Several stable dienones have been synthesized and are being tested as inhibitors of tyrosine-phenol lyase. Even more important applications of the principle involve the synthesis and testing of dienone analogues of the catecholamines and dihydroxyphenylalanine; the synthesis of such analogues presents a novel and unprecedented challenge. Studies have also been initiated to explore the synthesis of stable CH tautomers of histidine and histamine, for which we have already established a role in test-tube reactions and which we believe to be the true substrates in certain enzymatic processes. Recent results with histidine-ammonia lyase confirm our proposal for the involvement of a histidine CH-tautomer as the "true" substrate for this enzyme.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31115-09 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Functionalized Congeners of Bioactive Compounds

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. A. Jacobson	Research Chemist	LBC, NIDDK
Others:			
	P. J. M. van Galen	Visiting Fellow	LBC, NIDDK
	B. Fischer	Visiting Fellow	LBC, NIDDK
	X.-d. Ji	Visiting Associate	LBC, NIDDK
	N. Melman	Special Volunteer	LBC, NIDDK

COOPERATING UNITS (if any)

J. Baumgold (GW University); G. Stiles (Duke University); Y. Karton (Israel Inst. Biological Research, Nes Ziona, Israel); E. Heilbronn (Univ. Stockholm); P. Skolnick (IN, NIDDK).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Drug Receptor Interactions Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

2.2

PROFESSIONAL:

2.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) Human subjects (b) Human tissues x (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Recent work in our laboratory has demonstrated that certain drugs may be attached to well-defined "carrier" molecules and still retain the ability to bind to the receptor site and effect biological activity. This synthetic strategy for the attachment of drugs to carriers is termed the "functionalized congener" approach. The "carrier" molecule may be many times larger than the parent drug; indeed there is practically no maximum size limitation for a fully potent analog. Unlike the prodrug approach or the immobilization of drugs for slow release, the "functionalized congener" approach is designed to produce analogs for which no metabolic cleavage step is necessary for activation. Moreover, the attachment of the drug to a "carrier" such as a peptide may result in enhanced affinity at an extracellular receptor site and an improvement in the pharmacological profile of the parent drug.

Purine derivatives containing attached chains were developed as functionalized congeners for adenosine receptors. Reporter groups such as fluorescent dyes have been covalently attached resulting in receptor probes of relatively high affinity. Sites for chain derivatization on the structures of telenzepine and pirenzepine (useful drugs in treating stomach ulcers and as research tools for the brain), two selective muscarinic antagonists, have been located. In a series of amino alkyl derivatives, it was found that increasing the chain length enhances the potency of the derivative as a muscarinic antagonist. By incorporation of a phenyl isothiocyanate group, chemically reactive affinity labels for muscarinic receptors were developed. Other reporter groups included in the telenzepine series include biotin, p-aminophenylacetyl (for preparing radiotracers and photoaffinity labeling reagents), and fluorescent dyes fluorescein and tetramethylrhodamine (for locating the receptor sites microscopically and for binding assays that do not require the use of radioisotopes).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31116-05 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Prosthetic Groups for Labeling of Functionalized Drugs and Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: K. A. Jacobson Research Chemist LBC, NIDDK

COOPERATING UNITS (if any)

R. Eastman (NIDDK); M. Lesniak (NIDDK); M. Channing (NM-CC); G. Stiles (Duke University).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Drug Receptor Interactions Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

(a) Human subjects (a1) Minors (a2) Interviews
(b) Human tissues x (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The use of radioisotopes to label organic compounds for use in diagnostic nuclear medicine is well documented in the literature. It has been found that certain radiolabeled compounds will localize in the brain, heart, or in other target organs or tissues to a sufficient level to allow for imaging thereof. There has been increasing interest in finding compounds which will more effectively cross the blood-brain barrier, thus facilitating more efficacious imaging of the brain.

Binding sites for certain drugs in an animal or organ may be localized as a result of the synthesis of high specific activity radiolabeled analogs which have high affinity for that binding site. Prosthetic groups may be attached to a drug or other receptor ligand for the purpose of efficient and selective chemical capture of a particular radioisotope. Having developed functionalized congeners of theophylline and other drugs acting at adenosine receptors, we are now developing prosthetic groups for radioisotopes such as 18-F, 123-I, and 125-I, to be coupled to these functionalized drug molecules. The prosthetic groups contain amino or carboxylic groups which are to be condensed covalently to functionalized drugs to give conjugates of high affinity at a particular receptor, or drugs that bind the label irreversibly (trifunctional reagents). Using a general scheme, we have developed a radiolodinated xanthine derivative that contains an isothiocyanate group for covalent reaction with A1-adenosine receptors.

Positron emission tomography (PET) has been used for imaging receptors in the brain and other organs. A prosthetic group for chemical capture of 18-F requires rapid and efficient reaction and purification; since the half life is only 110 minutes. We are utilizing this approach to image insulin receptors in vivo.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31117-05 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Drugs Acting at Adenosine Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. A. Jacobson	Research Chemist	LBC, NIDDK
Other:	X.-d. Ji	Visiting Associate	LBC, NIDDK
	P. J. M. van Galen	Visiting Fellow	LBC, NIDDK
	M. Maillard	Special Volunteer	LBC, NIDDK
	N. Melman	Special Volunteer	LBC, NIDDK
	D. von Lubitz	Special Volunteer	LBC, NIDDK

COOPERATING UNITS (If any)

G. Stiles (Duke Univ.); R. T. Bartus (Cortex Pharmaceut.); K. Lee (Univ. Virginia); L. Belardinelli (U. Florida); R. A. Olsson (Univ. So. Florida); J. Baumgold (GW Univ.); A. P. IJzerman (Center for Bio-Pharmaceut. Sci., Leiden, The Netherlands).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Drug Receptor Interactions Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

2.9

PROFESSIONAL

2.9

OTHER

0

CHECK APPROPRIATE BOX(ES)

(a) Human subjects ☒ (b) Human tissues ☐ (c) Neither ☐
(a1) Minors ☐
(a2) Interviews ☐

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The extracellular adenosine receptor has a modulatory role in the nervous, circulatory, endocrine, and immunological systems. The prospect of harnessing these effects specifically for therapeutic purposes is attractive.

We have developed research tools for the characterization of adenosine receptors in vitro and in vivo. We have synthesized new drug analogues and elucidated structure activity relationships at receptor subtypes. Derivatives of adenosine with chemical modifications at the N6 and C-2 positions of the purine ring act as selective adenosine agonists. A1-agonists are being explored as cerebroprotective agents. To enhance brain uptake, prodrug schemes are being examined. APEC, an A2-selective adenosine amine congener served as the basis for a photoaffinity labeling reagent that allowed the first determination of the molecular weight of the receptor. Functionalized congeners of xanthines act as potent adenosine antagonists and are being developed as radioactive tracers for adenosine receptors and as affinity labels. Tritiated XAC (xanthine amine congener) was used to characterize the human striatal A2 adenosine receptor.

Since the two major subtypes of adenosine receptors have been cloned it has been possible to conduct molecular modeling of the receptor protein, based on sequence analyses and computerized energy minimizations. A hypothesis concerning the mode of binding of ligands to adenosine receptors has been derived. This hypothesis is consistent with pharmacological observations and site directed mutagenesis experiments, in which key histidyl residues have been replaced by other amino acids.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31118-03 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Bioindoles and Oxindoles as Medicinal and Diagnostic Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen

Chief, Section on Biochem. Mech.

NIDDK-LBC

Other: Yigal Fraenkel

IRTA Fellow (EOD 1/92)

NIDDK-LBC

COOPERATING UNITS (if any)

Sanford Markey, NIMH; P.A. Cohen, Univ. Brit. Columbia; Peter Kador, NEI; J. Flippen-Anderson, NRL, Washington, DC; R. Labroo, Seattle, WA.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.8

PROFESSIONAL:

0.8

OTHER:

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Radiiodinated melatonin (N-acetyl-5-methoxytryptamine) has been in use for some years as a tool in radioimmune assay. The iodination by ICl occurs at C-2 in very poor yield and produces a variety of other products, necessitating elaborate purification by HPLC. Since we had previously achieved the syntheses of 2-chloro- and 2-bromotryptophan in very good yield, we undertook a reexamination of the iodination problem. Initial efforts to achieve iodination of N-acetyltryptophan methyl ester were very disappointing. Far better results were obtained with the N-trifluoroacetyl derivative (65%) and further study showed that the introduction of all three halogens could be readily achieved without the need for the radical generators used in our earlier work. Further work demonstrated that effective halogenation depends on the degree of acidity of the acylamino NH group, with trifluoroacetyl being the most acidic of the series.

It now became obvious that replacement of the N-acetyl group of melatonin by N-trifluoroacetyl should improve the iodination yield considerably, and this alternative is now being explored. To our surprise, the N-trifluoroacetyl analogue had never been prepared and its biological activity, therefore, is not known. The compound will soon be evaluated for comparison in activity to the natural N-acetyl derivative. N-trifluoroacetylserotonin is also being prepared for parallel studies.

Several years ago, we made a concerted effort to obtain 2-fluoroindoles by halogen exchange with 2-bromoindoles, but were unsuccessful. Direct fluorination by use of new fluorinating agents have now been surprisingly successful and such compounds are anticipated to find application as affinity labels, analogues of peptide hormones and PET scanning reagents (since the introduction of radiolabel requires only one step).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31119-03 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Novel Amino Acids for Conformational and Stereochemical Constraints in Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Other:	Catherine Dong	Visiting Fellow	NIDDK-LBC
	Albinus D'Sa	Visiting Fellow (Term 1/92)	NIDDK-LBC

COOPERATING UNITS (If any)

J. Flippen-Anderson, NRL, Washington, DC; S. Vonhof, USUHS; W. Durckheimer, Frankfurt, Germany; V. Labroo, Seattle, WA.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.6

PROFESSIONAL:

0.6

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

The 13-membered cyclic peptide, H-Tyr-D-Orn-Phe-Asp-NH₂ (cyc-Orn-asp), is a potent mu-selective opioid peptide. Molecular modelling of this peptide has revealed considerable flexibility in the ring structure, making it difficult to identify a single conformation which may be biologically relevant. In order to restrict further the conformational mobility in this peptide and others, we have designed and synthesized a novel acid based on pyrrolidine (PDA). This amino acid, if used in place of Orn or Lys for side-chain-side-chain cyclization, is expected to reduce considerably the conformational flexibility in cyclization, is expected to reduce considerably the conformational flexibility in the macrocyclic ring of these peptides owing to the introduction of a bicyclic structure. Moreover, the additional asymmetric center in the pyrrolidine ring may introduce differential stereochemical constraints to the binding of diastereoisomeric peptides, derived from the corresponding diastereoisomeric PDA's. Thus, these peptides may act as probes for delineating the stereochemical topology of the receptor in the vicinity of the ring structure and may have useful biological properties and applications. Synthesis in the 2-pyrrolidinealanine series had been completed last year and parallel efforts in the 3-pyrrolidine series are almost finished.

Optically active amino acids are being used increasingly as synthons for the preparation of a variety of chiral compounds. PDA's can be expected to be excellent synthons for compounds such as pyrrolizidines with the stereochemistry already defined at two centers. Furthermore, these amino acids may possess antimicrobial or other antimetabolic activities. An efficient method for the synthesis of all four optically active stereoisomers of 2-PDA has been developed. These amino acids will be used for the preparation of novel bicyclic opioid peptide analogues.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31120-03

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Fluorinated Analogues of Bioactive Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Other:	Kenneth L. Kirk	Chief, Section on Drug Receptor Interactions	NIDDK-LBC
	Cyrus R. Creveling	Senior Investigator	NIDDK-LBC

COOPERATING UNITS (if any)

P.W. Schiller, Montreal, Canada; W. Durckheimer, Frankfurt, Germany; Shaw Chen, CH, LMG.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Fluorinated analogues of many bioorganic compounds have become important pharmacological and medicinal agents. The advantages of fluorine substitution arise from its small van der Waals radius (1.35 Å). As a result, fluorine bonded to a trigonal carbon is used effectively as a C-H replacement. Because of similar aliphatic C-F and C-O bond lengths (1.39 and 1.43 Å, respectively), the halogen bonded to a tetrahedral carbon has been used frequently as an OH surrogate, particularly in studies with nucleoside analogues. These steric similarities allow fluorinated compounds to mimic their nonfluorinated parents with respect to recognition by various biological macromolecules such as enzymes, transport proteins and receptors. On the other hand, the high electronegativity of fluorine (4.0) can drastically alter electron density distribution in the molecule which, in turn, affects pK values in neighboring groups and molecular dipole moments. All these altered physicochemical properties, as a result of fluorine substitution, can result in drastically modified biological properties such as potency, transport and receptor selectivity. Furthermore, recent advances in 19-F NMR and electron energy-loss spectroscopic techniques, and 18-F positron-emission tomography (18-F PET), have increased the significance and application of fluorinated molecules as biological markers and diagnostic agents.

We have used acetyl hypofluorite for fast, efficient and regiospecific introduction of fluorine into the phenolic ring of tyrosine within its peptides. By use of a different fluorinating agent, N-fluoropyridinium triflate, fluorination at the C-2 position of tryptophan has been achieved. Extension of this method to tryptophan-containing peptides is in progress. Studies are under way to determine whether different fluorinating agents, by virtue of differences in reaction mechanism, can achieve selective fluorination of different amino acids.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31121-02 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemistry and Biology of Novel Pyrimidine and Purine Nucleosides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen

Chief, Section on Biochem. Mech.

NIDDK-LBC

Other:

COOPERATING UNITS (if any)

H. Kimoto, Nagoya, Japan; M. Nishida, Nagoya, Japan; H. Sawada, Ibaraki, Japan; Shozo Fujii, Nagoya, Japan; E. DeClercq, Louvain, Belgium; W. Durckheimer, Frankfurt, Germany.

LAB/BRANCH

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Section on Biochemical Mechanisms

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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Uracil reacts specifically with bis(perfluoroalkanoxy)peroxides under radical conditions to give perfluoroalkyl derivatives at C-5. The latter compounds lose two fluorine atoms readily at pH 8-9 to form perfluoroalkyl ketones, which exist in equilibrium with tetrahedral adducts. Since the half-time for loss of fluorine is ca. 20 min at pH 8, these compounds are potential irreversible labels for pyrimidine recognition sites in vivo. Under these conditions, the perfluoroalkylpyrimidines are 3-5 times as reactive as trifluoromethylpyrimidines, whose strong antiviral effects have been thoroughly demonstrated. The same radical perfluoroalkylation can be achieved with uridines. Although the carbon-fluorine bond is significantly more stable in the nucleoside series, conversion to ketones can also be achieved with sulphydryl or cyanide catalysis. These ketones are reducible to secondary alcohols with sodium borohydride. The inductive effects of multiple fluorine atoms renders the alcohols highly acidic and readily capable of forming strong internal hydrogen bonds to functions at C-4. We anticipate that such hydrogen bonding will interfere with the intermolecular hydrogen bonding needed for effective base pairing in polynucleotide strands. Thus, such compounds may block cell division and act as antiviral and anticancer agents. Furthermore, hydrogen bonding to this position has been found essential for the action of PRPP synthetase.

Analogous perfluoroalkyl groups have been introduced into purines, primarily at C-8; these compounds provide an additional series of "preaffinity" labels. Our earlier studies on the chemistry of perfluoroalkylimidazoles indicated that such a group at C-8 of a purine should undergo loss of fluorine at physiological pH and provide, in vivo, a very reactive functional group capable of bonding to a protein or nucleic acid nucleophile. Current efforts involve the attachment of dideoxyribose moieties to these purine analogues. All of these compounds also have the potential of acting as inhibitors of cytidine deaminase; biological evaluations are in progress.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31122-02 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Antimalarial Agents Based on Bioheterocycles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Louis A. Cohen	Chief, Section on Biochem. Mech.	NIDDK-LBC
Other:	Rahul Jain	Visiting Fellow (EOD 12/91)	NIDDK-LBC
	Ellen Goldberg	IRTA Summer Student	NIDDK-LBC

COOPERATING UNITS (if any)

S. Avaramovivi and S. Sarel, Jerusalem, Israel; W. Durckheimer, Frankfurt, Germany.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

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Section on Biochemical Mechanisms

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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.8

PROFESSIONAL:

0.7

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Our new amino acid, 2-iodo-L-histidine, is a potent antimalarial agent but undergoes rapid deiodination in vivo by cysteine or glutathione. The mechanisms of deiodination requires prior protonation of the imidazole ring; thus, 2,4-diiodohistidine is deiodinated at 1/10 the rate of 2-iodohistidine, because the second halogen reduces ring basicity to a significant degree. Unfortunately, the second iodine introduces extra steric bulk, which may be the reason the diiodo compound is biologically inactive. But the same reduction in basicity can be achieved with much smaller groups, and this fact provides a lead for new analogs. 4-(Trifluoromethyl)histidine has been iodinated at C-2, and the large trifluoromethyl group converted to the very small and very electronegative cyano group by exposure to aqueous ammonia. While histidine and its protected derivatives undergo bromination and iodination with extreme ease, chlorination has always proved almost impossible to achieve. We have found that "carbonylcyclohistidine", the bicyclic urea obtained by reaction of histidine ester with carbonyldiimidazole, undergoes chlorination very easily to produce both the mono and dichloro derivatives. Iodination of the monochloro derivative, followed by acid hydrolysis, provides the desired 4-chloro-2-iodo-L-histidine. As expected, 2-iodo-histidines containing strong electronegative groups at C-4 do not undergo rapid deiodination by mercaptans.

Examination of space-filling models reveals that 2-iodohistidine has a width corresponding exactly to the diameter of the erythrocyte membrane channel, as estimated from the diffusion rates of small molecules. The same dimension can be found in metabolically stable molecules, such as 1-isopropyl and 2-isopropylhistidine. A program was initiated to develop general synthetic methods for these previously inaccessible ring-alkylated histidines (and histamines). Several novel approaches have proved successful, and extensive series of 1-alkyl and 2-alkylhistidines are being prepared for screening.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 32001-1 LBC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Development of Multifunctional Chemotherapeutic Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name title laboratory and institute affiliation)

PI: B. Vithal Shetty Guest Researcher LBC, NIDDK

Others: K. L. Kirk Chief, Section on Drug-Receptor Interactions LBC, NIDDK

COOPERATING UNITS (if any)

LAB BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Drug-Receptor Interactions

INSTITUTE AND LOCATION

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TOTAL STAFF YEARS

0.4

PROFESSIONAL

0.4

OTHER

CHECK APPROPRIATE BOX(ES):

- (a) Human subjects (b) Human tissues (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided)

A novel approach to the design of new antimicrobial agents has been developed. The overall purpose of the program is the rational design and synthesis of compounds that have combined anti-bacterial, anti-viral and anti-fungal activity. Specifically targeted in the rational design are enveloped viruses such as herpes viruses and HIV. These compounds have novel structural features which include a dimeric attachment of a known hydrophobic antimetabolite (for example, aminoadamantane analogues) through a very hydrophilic bridge. These structural features combine to provide the potential for a novel mechanism for attachment of the toxic moiety to microbial organisms. Thus, the hydrophilic portion of the molecule has a high affinity for glycoprotein components of the microbial cell wall. This affinity will deliver the toxic moieties to the cell surface, providing a mechanism for efficient activity. For example, the potential exists for inhibition of replication of such viruses as HIV and, through the attachment of the toxic molecule to the viral coat, a mechanism for killing the virus. Compounds in the series were found to have potent activity against gram positive and gram negative bacteria, fungi, yeast and enveloped viruses. Such combined activity potentially could be extremely useful in treatment of immunosuppressed patients.

The Laboratory of Molecular Biology has as its principal goal the understanding of biological processes at the molecular level. The research program involves a broad range of experimental approaches to problems in molecular genetics, the regulation of gene expression in prokaryotes and eukaryotes, and the structures of nucleic acids and proteins. Current research includes studies of the organization of DNA and proteins within the eukaryotic nucleus, studies of the molecular mechanisms for establishing and maintaining stable states of gene expression during embryonic development, studies of site-specific recombination mechanisms in a variety of systems including retroviruses, bacteriophage, and the genes of the immune system, and studies of DNA supercoiling and its regulatory effects as well as studies of the molecular mechanisms used by enzymes responsible for supercoiling. More direct structural research includes studies on the chemistry, structures and interactions of polynucleotides, calorimetric studies of nucleic acids and proteins, investigations of molecular crowding in biological systems, and a large number of crystallographic studies of the structures of enzymes, viral proteins, and immunoglobulins. Significant advances have been made during the past year in all of these areas.

Chromatin Structure and Function

We have continued our studies of chromatin structure in the neighborhood of expressed genes. The globin gene family in chicken erythroid cells serves as a model system in which it is possible to study the mechanisms associated with regulation of the individual members of the family during erythroid development. We have extended our studies of the properties of the general erythroid-specific factor GATA-1. We have shown that a small peptide containing one 'finger' region of GATA-1 is capable of binding tightly and specifically to its target DNA. Furthermore, this region can bind as either a zinc or iron complex, suggesting that in some organisms, members of this family might be iron proteins. We have extended our studies of stage-specific erythroid expression to the chicken embryonic ρ -globin gene. We find that there is a considerable decrease in the nuclear concentration of two trans-acting factors that we can show are critical to stage-specific expression of this gene, and that may account in large part for the difference in the behavior of the promoter in primitive and definitive lineage cells. We also have continued studies of the relationship between transcription and chromatin structure. We devised a method of determining the fate of nucleosome cores during transcription of nucleosome-covered DNA. We can show that the cores are displaced by the passage of SP6 RNA polymerase, but that they reform at sites elsewhere on the template. From the distribution of these sites, we eliminate several proposed models for propagation of transcription on chromatin templates, and propose our own. The results are consistent with a mechanism we had proposed earlier.

Studies on the Mechanism of Genetic Recombination

The major objective of this project is to uncover the molecular mechanisms responsible for a variety of genetic rearrangements. The transposition-replication reaction of bacteriophage Mu is studied under this project as a model system.

Critical steps in Mu transposition are a pair of DNA cleavages and strand transfers which

generate a branched DNA intermediate. Efficient formation of this intermediate requires the phage-encoded MuA and MuB proteins and the E. coli-encoded HU and IHF proteins, ATP and Mg^{++} . The MuA protein interacts with two distinct types of DNA sequences, one type of sequence is from the ends of the Mu genome while the other lies at an internal site within the Mu operator. These interactions with the donor DNA lead to formation of a stable protein-DNA complex in which the two Mu ends are synapsed by a tetramer of MuA. Next, a pair of single strand cuts are made to expose the 3' ends of the Mu sequence. This cleaved donor DNA remains tightly associated with the MuA tetramer and this complex efficiently captures a second "target" DNA molecule provided it is bound by MuB protein. A staggered cut is introduced into the target DNA and the two 5' ends are joined to the 3' ends of the Mu end sequences in a concerted DNA cutting and joining reaction. Evidence has been obtained that this reaction takes place by one-step transesterification mechanism.

The MuB protein, an ATPase, selectively stimulates utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The MuB protein dissociates preferentially from DNA molecules bound by MuA protein in a process that depends on ATP hydrolysis. Kinetic aspects of this energy transduction system have been studied.

Studies of the Mechanism of Retroviral DNA Integration

Integration of a DNA copy of the retroviral genome into a chromosome of the host cell is an essential step in the retroviral replication cycle. The objectives of this project are to understand the detailed molecular mechanism of the integration reaction and to facilitate the development of inhibitors that block this step in the replication cycle.

We have previously shown that the viral integrase protein carries out the central steps of the integration reaction in vitro. Our recent work has continued to focus on the biochemical activities of the HIV integrase protein. Integrase catalyzes two distinct reactions: site-specific cleavage of two nucleotides from the 3' ends of the viral DNA and a subsequent reaction that inserts the resulting processed ends into a target DNA. Stereochemical analysis of these reactions supports the view that they both proceed by a one-step mechanism, not involving a covalent intermediate between integrase and the DNA substrate. We have analyzed the functional organization of HIV integrase by expressing and purifying mutant proteins with changes at selected amino acid positions, or deletions extending from the N- or C-terminus. Substitution of conserved amino acids in a central part of the protein that is highly conserved among retroviral integrases abolished catalytic activity, suggesting a key role for this part of the protein in catalysis. In contrast, a conserved motif near the N-terminus is not essential for catalysis, but may be important for protein-DNA or protein-protein interactions.

Retroviral DNA made by reverse transcription after infection of a sensitive cell exists as part of a large nucleoprotein complex, derived from the viral core. Although purified integrase protein carries out the DNA cutting and joining steps of integration in vitro, some aspects of the reaction are not efficiently reproduced with integrase alone, but are

reproduced when *in vitro* reactions are carried out with complexes isolated from infected cells. We are analyzing such complexes, isolated from cells infected with Moloney murine leukemia virus, to determine the factors that contribute to this greater fidelity.

Studies of Immunoglobulin Gene Rearrangement

Previous work on antigen receptor gene rearrangement (V(D)J recombination) has described the signal sequences that are recognized and the recombined products, but there was no information about the progress of the reaction. It has now become possible to detect broken DNA molecules that may be intermediates in this recombination.

In the thymus of young mice, where the T cell receptor δ locus (TCR δ) is being actively rearranged, double-strand breaks at the D δ 2 and J δ 1 loci are readily detected by Southern blotting. About 2% of the DNA's is broken - this is a remarkably high proportion. Broken molecules are found only in thymus and not in other tissues where TCR δ is not undergoing rearrangement. Of the two different ends that should be generated by each break, only one (the signal end) could be detected. The coding ends may be joined more rapidly. These results provide the first direct support for a breakage - reunion model of V(D)J recombination.

In related work, a physical assay has been developed that allows the direct detection of V(D)J joining in plasmid substrates, without the need for a biological enrichment.

Studies of Functions Involved in Genetic Recombination

New results have been obtained on enzymes that alter DNA supercoiling, and on the role of supercoiling in controlling transcription.

It was previously shown that ATP binding site of DNA gyrase can be labeled by an ATP affinity analog. However, two residues in the Gyr B subunit were equally labeled, lysine-103 and lysine-110. Mutations of these sites now show that lysine-103 is essential, but lysine-110 is not.

A new topoisomerase has been isolated from a thermophilic archaebacterium, with properties much closer to eukaryotic topoisomerases than to other bacterial enzymes, because it is able to relax positively as well as negatively supercoiled DNA, and to work in the absence of divalent metal ions.

Further studies of DNA relaxation - stimulated transcription in *E. coli* have shown that promoter sites with this property also lead to transcription that reads through termination signals. This readthrough is not found when using purified RNA polymerase *in vitro*, indicating the involvement of other factors.

Nonheritable Antibiotic Resistance

We previously reported that salicylate (SAL) decreases the LD₅₀ of Cd⁺⁺ in *Escherichia coli* by 3- to 4-fold. In order to determine the bacterial functions that are required for this synergy, we selected mutants (called Sci-) whose susceptibility to Cd⁺⁺ was not enhanced

by the presence of SAL. Surprisingly, 5/14 of these Sci- mutants were also cysteine auxotrophs. The mutations responsible for the auxotrophies were located in the *cysB* gene as determined by Hfr mapping and by complementation with a plasmid carrying wild-type *cysB*. The *cysB* mutations were shown to be responsible for the Sci- phenotypes since mutants that became Cys+ by recombination, complementation or reversion simultaneously became Sci+. Furthermore, *cysB* mutants that had previously been isolated solely on the basis of their requirement for cysteine were also found to be Sci-. Thus, a function of *cysB* is necessary for the potentiation of Cd⁺⁺ susceptibility by SAL. Since CysB is a regulatory protein required for transcriptional activation of the several operons of the *cysB* regulon, *cysB* mutants do not express at least 12 other *cys* genes needed to synthesize cysteine. Mutations in 5 of these *cys* genes did not result in a Sci- phenotype. Thus, the Sci- phenotype is a property of only particular *cys* mutations and is not due simply to the inability to synthesize cysteine. So far, the only other *cys* mutations that render cells Sci- were found in *cysE*, a gene whose function is not under *cysB* control but is required for activation of the CysB protein.

Since the transcription of *cysB* is regulated by supercoiling, we examined the sensitivity to Cd⁺⁺ of *topA* mutants whose DNA is highly supercoiled. These cells were found to be hypersensitive to Cd⁺⁺. Suppression of the *topA* mutation abolished the hypersensitivity. Cd⁺⁺ resistant mutants were then isolated from the *topA* cells: 3/5 were *cysB* mutants and were Sci-. Thus, these effects of SAL and supercoiling have a common requirement for *cysB*. However, SAL did not affect the transcription of either *cysB* or *cysE* as shown by studies with appropriate *cysB/E-lacZ* fusion strains. Further studies are necessary to determine whether SAL affects the activity of CysB protein. We conclude that a function under *cysB* control (which may or may not be involved in cysteine biosynthesis) is required for the potentiation of Cd⁺⁺ sensitivity by SAL and supercoiling.

E. coli Genes Whose Expression is Altered by Salicyl Alcohol

Salicylates, like heat shock and UV damage, induce a panoply of changes in *E. coli* including (but not limited to): the induction of resistance to certain classes of antibiotics and sensitivity to others; alteration in outer membrane protein composition; and increase sensitivity to Cd⁺⁺. Some of these effects may be of the weak acid variety, but the majority are not as they are also induced by salicyl alcohol. In an attempt to dissect and define the action of salicyl alcohol on *E. coli*, we have constructed two libraries of 10⁵ independent transpositional events, each containing the *lac Z* gene (with and without translational initiation signals) inserted at random about the *E. coli* genome. We are currently in the process of screening these libraries for transpositional events in which the *lac Z* gene is induced or repressed by salicyl alcohol and in which the resistance of nalidixic acid is not increased by salicyl alcohol. A number of potential mutants have been identified and we are beginning to map and characterize them.

Thermal Measurements of Biomolecular Systems

We have re-examined the thermodynamic parameters we have obtained for sequence-nonspecific DNA association and sequence-specific DNA associations with Cro protein. The association of Cro protein with nonspecific DNA at 15°C has a ratio of $\Delta S^\circ/\Delta G^\circ$ close to the value of the temperature dependence of the dielectric constant of water which is as predicted by simple electrostatic theories of ion hydration (Born) and ion-association (Bjerrum). Plots of ΔH vs. ΔC_p and ΔS° vs. ΔC_p for the twenty association reactions studied fall into two characteristic correlation groups. These results suggest that there are at least two distinct conformational subclasses in specific Cro-DNA complexes, which are stabilized by different combinations of enthalpic and entropic contributions. (With Y.

Takeda & C.P. Mudd).

In the maturation of the capsid of bacteriophage T4, gp23, the protein primarily comprising the precursor shell, undergoes proteolysis to gp23 and the particles expand 15-20%. We have now investigated the intermediate states of capsid maturation; the cleaved/unexpanded state and the uncleaved/expanded state, by differential scanning calorimetry (DSC) and cryo-electron microscopy. Combining these and earlier results, we conclude that the expansion of the cleaved precursor is the major stabilization in T4 capsid maturation. (With A.C. Steven).

We have completed a detailed thermodynamic analysis of ligand-induced multiphasic thermal protein denaturation. This treatment shows that the multiple peaks observed in DSC experiments, when ligand is in short supply, arises from the substantial increase in the free ligand concentration due to ligand released by unfolding protein. It is shown for the case of a protein having two ligand binding sites, that the observed bimodal DSC thermogram arises primarily from the contribution of partially liganded species rather than from ligand-free and fully saturated native species as is commonly believed. (With A. Shrake).

Influences of Macromolecular Crowding on Biochemical Systems

The high concentrations of macromolecules within cells result in large excluded volumes, potentially causing very large shifts in rates and equilibria of reactions involving other macromolecules. Prior studies of such macromolecular crowding effects on DNA by ourselves and others have been restricted to empirical studies of model systems, leaving unanswered basic questions about DNA function under crowded conditions. We have addressed two of those questions.

First we have studied the effective volume of DNA and related materials in crowding interactions. The two-phase distribution assay for volume occupancy described earlier was used to determine the effective volumes in crowding interactions of a series of small double- and single-stranded pieces of DNA. Their behavior is fully consistent with a simple theoretical treatment, the available volume model, used for predicting excluded volume effects. These results are "in press" in Biopolymers.

Second, we are currently measuring the crowding effects of actual cellular material, cytoplasmic extracts from E. coli, on a DNA test system. The test system measures the rate of in vitro cohesion of restriction fragments of λ DNA. Preliminary results indicate accelerations of at least 1-2 orders of magnitude.

Chemical and Structural Investigations of Nucleic Acids and Related Molecules

We have shown that the generally accepted DNA triple helix structure proposed by Arnott and Selsing (1976) is incorrect in important respects and have obtained a greatly improved model supported by clear experimental and molecular modeling evidence. In other recent work on triple helices we have shown that pyrimidine third strands must be parallel to the purine strand (Hoogsteen pairing). Proposed reverse Hoogsteen structures cannot be observed experimentally and are stereochemically unsatisfactory. With purine third strands (A to A, G to G) the opposite conclusion was reached. Triplexes can be formed in which two purine strands are antiparallel but cannot be formed if they are

parallel.

The human telomere sequence TTAGGGTTAGGG forms a single structure as a 12mer and a 24mer under defined conditions, in contrast to most of the sequences we and others have examined. By modeling we have obtained an attractive proposed structure in which all of the bases are paired. We have synthesized an analogous 9mer for attempted crystallization and NMR study.

Though the DNA triple helices dC:dG:C⁺ are often referred to in the literature, we have found that they cannot be formed because of competing formation of the very stable poly dC acid helix. The same is true when dC₂₀ or dC₈ are used as potential third strands.

We have prepared a Hoogsteen double helix with parallel strands using only natural nucleic acid bases. Infrared spectra in the conformationally sensitive region from 8000 to 900 cm⁻¹ show that the backbone conformation of the duplex is virtually identical to that of the corresponding triple helix. We have also prepared a "parallel" DNA with reverse Watson-Crick pairing by attaching C₂ "C clamps" to assure parallel structure. With certain sequences the structure remains parallel even without the terminal C's.

Aids Related Proteins: Structure and Function

The crystal structure of Transforming Growth Factor Beta II has been determined (see below).

The HIV integration protein has been the subject of isolation and purification procedures to establish methods for the preparation of adequate protein for crystallization trials. Solubility problems associated with the original HIV I preparation have led to a search for related proteins that are more suitable for crystallization.

Three-Dimensional Structures of Cytokines, Receptors and Immune System Proteins

The crystal structure of Transforming Growth Factor-beta (TGF-beta 2) has been determined at 2.1Å resolution. The molecule is a homodimer, with each subunit having an unusual structure of a kind not previously observed in other proteins. Comparison with other members of the TGF-beta family and with the members of the superfamily such as activins and inhibins indicate that they probably adopt very similar structures. A preliminary identification of the receptor binding site has been made.

The crystal structure of the complex of the FAB HyHEL-5 with chicken lysozyme has been refined. Also, the structure of the complex of this antibody with a mutant lysozyme has been determined. In the mutant an arginine present in the wild type has been replaced by a lysine (R68K). A comparison of the mutant and the wild type has been made in order to explain the rather large effect of this conservative mutation on the affinity.

Enzyme Structure

The multi-enzyme complex, tryptophan synthase from salmonella typhimurium, has been further analyzed by X-ray diffraction. New very high resolution data have been measured for a mutant of the beta subunit, in the external aldimine form with the amino acids serine

and tryptophan, and these structures have been refined. This analysis provides new information about the disposition of residues in the beta active site and their interaction with the intermediates of the beta reaction.

Inhibitor complexes of rhizopus pepsin have been analyzed by X-ray diffraction with a view to obtaining more information concerning the mechanism of action. One of these provides a model for the tetrahedral intermediate of the transition state. Examination of the contact distances of this complex provides strong support for the previously proposed mechanism of action.

A new method has been presented for the joint refinement of X-ray and NMR data. This method can demonstrate that only very small differences between the structures result from the procedures used, and can highlight real conformational differences caused by physical differences such as crystal packing.

Structural Studies of Molecular Recognition

X-ray diffraction data have been collected from crystals of the Fab of CC49, a murine monoclonal antibody against solid adenocarcinoma, and molecular replacement analysis of the crystal structure is in progress.

The known structures of Fabs have been analyzed to determine which framework residues need to be preserved in the 'humanization' of xenogeneic antibodies by CDR-grafting.

A model of the extracellular portion of the alpha-subunit of the human high-affinity IgE receptor has been built.

The binding of various viral and self peptides to the murine class I MHC antigen, H-2Ld, has been modelled.

Study of the Potential Use of Catalytic Antibodies Against AIDS

Three peptidic transition-state analogs have been proposed from highly-conserved polypeptide segments of the HIV-1 envelope glycoprotein and two of these analogs have been synthesized and used as immunogens for the production of murine monoclonal antibodies. Several hundred hybridomas have been shown to bind to the transition-state analog used for immunization and several of these show differential binding to the transition-state analog vs the original-sequence peptide.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 33000-26 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Functions Involved in Genetic Recombination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Martin Gellert, Ph.D.	Chief, Section on Metabolic Enzymes	LMB/NIDDK
Sergei Kozyavkin, Ph.D.	Visiting Associate	LMB/NIDDK
Regis Krah, Ph.D.	IRTA Fellow	LMB/NIDDK
Mary H. O'Dea	Research Chemist	LMB/NIDDK

COOPERATING UNITS (If any)

Dr. Rolf Menzel, Bristol-Myers-Squibb, Princeton, N.J.

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Laboratory of Molecular Biology/NIDDK

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.2

PROFESSIONAL:

3.2

OTHER:

0

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

New results have been obtained on enzymes that alter DNA supercoiling, and on the role of supercoiling in controlling transcription.

It was previously shown that ATP binding site of DNA gyrase can be labeled by an ATP affinity analog. However, two residues in the Gyr B subunit were equally labeled, lysine-103 and lysine-110. Mutations of these sites now show that lysine-103 is essential, but lysine-110 is not.

A new topoisomerase has been isolated from a thermophilic archaebacterium, with properties much closer to eukaryotic topoisomerases than to other bacterial enzymes, because it is able to relax positively as well as negatively supercoiled DNA, and to work in the absence of divalent metal ions.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 33001-8 LMB

PERIOD COVERED

October 30, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Immunoglobulin Gene Rearrangement

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Martin Gellert, Ph.D.	Chief, Section on Metabolic Enzymes	LMB/NIDDK
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Joseph Menetski, Ph.D.	Guest Researcher	LMB/NIDDK
David Roth, MD., Ph.D.	Special Volunteer	LMB/NIDDK
Moshe Sadofsky, MD., Ph.D.	Special Volunteer	LMB/NIDDK

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5.8

PROFESSIONAL:

5.8

OTHER:

0

CHECK APPROPRIATE BOX(ES):

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- ☐ (a1) Minors
- ☐ (a2) Interviews

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In related work, a physical assay has been developed that allows the direct detection of V(D)J joining in plasmid substrates, without the need for a biological enrichment.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 33006-14 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Mechanism of Genetic Recombination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

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4

PROFESSIONAL:

4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 34001-27 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Gary Felsenfeld, Ph.D.	Chief, Section on Physical Chemistry	LMB/NIDDK
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Gretchen Gibney, Spec. Vol.	LMB/NIDDK	Hannah Gould, Expert
Joseph Knezetic, Spec. Vol.	LMB/NIDDK	Mark Minie, Staff Fellow
Cecilia Trainor, IRTA	LMB/NIDDK	Henryk Eisenberg, V. Sc.
Rodolfo Ghirlando, V. Sc.	LMB/NIDDK	Vasily Studitsky, V. Sc.
Joseph Grasso, Visiting Asso.	LMB/NIDDK	Emery Bresnick, V. Fellow
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COOPERATING UNITS (if any)

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Dept. of Biophysics, King's College (Robert Hannon) - NICHD, NIH (Heiner Westphal)

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10.5

PROFESSIONAL:

10.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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☐ (a1) Minors
☐ (a2) Interviews

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 34002-28 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enzyme Structure

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

David R. Davies, Ph.D.	Chief, Sec. on Molecular Structure	LMB/NIDDK
Gerson H. Cohen, Ph.D.	Research Chemist	LMB/NIDDK
C. Craig Hyde, Ph.D.	Special Volunteer	IRP/LSBR
Kevin D. Parris, Ph.D.	Staff Fellow	LMB/NIDDK
Boaz Shaanan, Ph.D.	Visiting Scientist	LMB/NIDDK

COOPERATING UNITS (if any)

Edith Miles, NIDDK

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TOTAL STAFF YEARS

3.3

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The multi-enzyme complex, tryptophan synthase from salmonella typhimurium, has been further analyzed by X-ray diffraction. New very high resolution data have been measured for a mutant of the beta subunit, in the external aldimine form with the amino acids serine and tryptophan, and these structures have been refined. This analysis provides new information about the disposition of residues in the beta active site and their interaction with the intermediates of the beta reaction.

Inhibitor complexes of rhizopus pepsin have been analyzed by X-ray diffraction with a view to obtaining more information concerning the mechanism of action. One of these provides a model for the tetrahedral intermediate of the transition state. Examination of the contact distances of this complex provides strong support for the previously proposed mechanism of action.

A new method has been presented for the joint refinement of X-ray and NMR data. This method can demonstrate that only very small differences between the structures result from the procedures used, and can highlight real conformational differences caused by physical differences such as crystal packing.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 34003-24 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Three-Dimensional Structures of Cytokines, Receptors and Immune System Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

David R. Davies, Ph.D.	Chief, Sec. on Molecular Structure	LMB/NIDDK
Christina Brown, Ph.D.	Visiting Associate	LMB/NIDDK
Susan Chacko, Ph.D.	Visiting Fellow	LMB/NIDDK
Gerson H. Cohen,	Research Chemist	LMB/NIDDK
Sun Daopin,	Visiting Associate	LMB/NIDDK

COOPERATING UNITS (if any)

Karl Piez, Fogarty Center
Yasushi Ogawa, Celltrix Corp., California

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

3

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type Do not exceed the space provided.)

The crystal structure of Transforming Growth Factor-beta (TGF-beta 2) has been determined at 2.1Å resolution. The molecule is a homodimer, with each subunit having an unusual structure of a kind not previously observed in other proteins. Comparison with other members of the TGF-beta family and with the members of the superfamily such as activins and inhibins indicate that they probably adopt very similar structures. A preliminary identification of the receptor binding site has been made.

The crystal structure of the complex of the FAB HyHEL-5 with chicken lysozyme has been refined. Also, the structure of the complex of this antibody with a mutant lysozyme has been determined. In the mutant an arginine present in the wild type has been replaced by a lysine (R68K). A comparison of the mutant and the wild type has been made in order to explain the rather large effect of this conservative mutation on the affinity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK35000-28 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemical and Structural Investigations of Nucleic Acids and Related Molecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

H. Todd Miles, Ph.D.	Chief, Section on Organic Chemistry	LMB/NIDDK
F. B. Howard, Ph.D.	Research Chemist	LMB/NIDDK
J. Frazier	Research Chemist	LMB/NIDDK
Keliang Liu, Ph.D.	Visiting Fellow	LMB/NIDDK

COOPERATING UNITS (if any)

Girjesh Govil, TIFR, India / Philip Ross LMB/NIDDK
V. Sasisekharan, Visiting Scientist; Indian Instit. of Science, Bangalore, India
C-O. Chen, Biotechnology Institute, Shanghai, China

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Organic Chemistry

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

5

PROFESSIONAL:

5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have shown that the generally accepted DNA triple helix structure proposed by Arnott and Selsing (1976) is incorrect in important respects and have obtained a greatly improved model supported by clear experimental and molecular modeling evidence. In other recent work on triple helices we have shown that pyrimidine third strands must be parallel to the purine strand (Hoogsteen pairing). Proposed reverse Hoogsteen structures cannot be observed experimentally and are stereochemically unsatisfactory. With purine third strands (A to A, G to G) the opposite conclusion was reached. Triplexes can be formed in which two purine strands are antiparallel but cannot be formed if they are parallel.

The human telomere sequence TTAGGGTTAGGG forms a single structure as a 12mer and a 24mer under defined conditions, in contrast to most of the sequences we and others have examined. By modeling we have obtained an attractive proposed structure in which all of the bases are paired. We have synthesized an analogous 9mer for attempted crystallization and NMR study.

Though the DNA triple helices dC:dG:C⁺ are often referred to in the literature, we have found that they cannot be formed because of competing formation of the very stable poly dC acid helix. The same is true when dC₂₀ or dC₆ are used as potential third strands.

We have prepared a Hoogsteen double helix with parallel strands using only natural nucleic acid bases. Infrared spectra in the conformationally sensitive region from 8000 to 900 cm⁻¹ show that the backbone conformation of the duplex is virtually identical to that of the corresponding triple helix. We have also prepared a "parallel" DNA with reverse Watson-Crick pairing by attaching C₂ "C clamps" to assure parallel structure. With certain sequences the structure remains parallel even without the terminal C's.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36003-8 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nonheritable Antibiotic Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

J.L. Rosner, Ph.D. Research Biologist LMB/NIDDK

Reeta Goel, Ph.D. Visiting Associate LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We previously reported that salicylate (SAL) decreases the LD₅₀ of Cd⁺⁺ in *Escherichia coli* by 3- to 4-fold. In order to determine the bacterial functions that are required for this synergy, we selected mutants (called Sci-) whose susceptibility to Cd⁺⁺ was not enhanced by the presence of SAL. Surprisingly, 5/14 of these Sci- mutants were also cysteine auxotrophs. The mutations responsible for the auxotrophies were located in the *cysB* gene as determined by Hfr mapping and by complementation with a plasmid carrying wild-type *cysB*. The *cysB* mutations were shown to be responsible for the Sci- phenotypes since mutants that became Cys+ by recombination, complementation or reversion simultaneously became Sci+. Furthermore, *cysB* mutants that had previously been isolated solely on the basis of their requirement for cysteine were also found to be Sci-. Thus, a function of *cysB* is necessary for the potentiation of Cd⁺⁺ susceptibility by SAL. Since CysB is a regulatory protein required for transcriptional activation of the several operons of the *cysB* regulon, *cysB* mutants do not express at least 12 other *cys* genes needed to synthesize cysteine. Mutations in 5 of these *cys* genes did not result in a Sci- phenotype. Thus, the Sci- phenotype is a property of only particular *cys* mutations and is not due simply to the inability to synthesize cysteine. So far, the only other *cys* mutations that render cells Sci- were found in *cysE*, a gene whose function is not under *cysB* control but is required for activation of the CysB protein.

Since the transcription of *cysB* is regulated by supercoiling, we examined the sensitivity to Cd⁺⁺ of *topA* mutants whose DNA is highly supercoiled. These cells were found to be hypersensitive to Cd⁺⁺. Suppression of the *topA* mutation abolished the hypersensitivity. Cd⁺⁺ resistant mutants were then isolated from the *topA* cells: 3/5 were *cysB* mutants and were Sci-. Thus, these effects of SAL and supercoiling have a common requirement for *cysB*. However, SAL did not affect the transcription of either *cysB* or *cysE* as shown by studies with appropriate *cysB*/*E-lacZ* fusion strains. Further studies are necessary to determine whether SAL affects the activity of CysB protein. We conclude that a function under *cysB* control (which may or may not be involved in cysteine biosynthesis) is required for the potentiation of Cd⁺⁺ sensitivity by SAL and supercoiling.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK36101-16LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Energy Conversion in Biology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator: (Name, title, laboratory, and institute affiliation))

PI: Yi-der Chen Research Chemist LMB/NIDDK

Others: Robert J. Rubin Special Volunteer LMB/NIDDK

COOPERATING UNITS (if any)

Shahid Khan, Albert Einstein College of Medicine, NY, NY 10461
Aydin Tozeren, Catholic University, Washington, D.C. 20064

LABORATORY

Laboratory of Molecular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard, unabbreviated type. Do not exceed the space provided.)

Researchers transferred to Laboratory of Chemical Physics.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01DK36102-21LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Statistical Thermodynamics of Protein and Polynucleotide Systems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator (Name, title, laboratory, and institute affiliation).)

PI: Yi-der Chen Research Chemist LMB/NIDDK

COOPERATING UNITS (if any)

J. Chalovich, University East Carolina, North Carolina

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES.

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Researcher transferred to Laboratory of Chemical Physics.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36104-11 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thermal Measurements of Biomolecular Systems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Philip D. Ross, Ph.D.	Research Chemist	LMB/NIDDK
A.C. Steven, Ph.D.	Visiting Scientist	LPB/NIAMS
S. Shrake, Ph.D.	Research Chemist	DBBP/CPB
Y. Takeda, Ph.D.	Research Chemist	FCRF/NCI
C.P. Mudd, Ph.D.	Engineer	BEIP/RR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK NIH Bethesda, Maryland 20892

TOTAL STAFF YEARS

2

PROFESSIONAL

2

OTHER

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have re-examined the thermodynamic parameters we have obtained for sequence-nonspecific DNA association and sequence-specific DNA associations with Cro protein. The association of Cro protein with nonspecific DNA at 15°C has a ratio of $\Delta S^\circ/\Delta G^\circ$ close to the value of the temperature dependence of the dielectric constant of water which is as predicted by simple electrostatic theories of ion hydration (Born) and ion association (Bjerrum). Plots of ΔH vs. ΔC_p and ΔS° vs. ΔC_p for the twenty association reactions studied fall into two characteristic correlation groups. These results suggest that there are at least two distinct conformational subclasses in specific Cro-DNA complexes, which are stabilized by different combinations of enthalpic and entropic contributions. (With Y. Takeda & C.P. Mudd).

In the maturation of the capsid of bacteriophage T4, gp23, the protein primarily comprising the precursor shell, undergoes proteolysis to gp23 and the particles expand 15-20%. We have now investigated the intermediate states of capsid maturation; the cleaved/unexpanded state and the uncleaved/expanded state, by differential scanning calorimetry (DSC) and cryo-electron microscopy. Combining these and earlier results, we conclude that the expansion of the cleaved precursor is the major stabilization in T4 capsid maturation. (With A.C. Steven).

We have completed a detailed thermodynamic analysis of ligand-induced multiphasic thermal protein denaturation. This treatment shows that the multiple peaks observed in DSC experiments, when ligand is in short supply, arises from the substantial increase in the free ligand concentration due to ligand released by unfolding protein. It is shown for the case of a protein having two ligand binding sites, that the observed bimodal DSC thermogram arises primarily from the contribution of partially liganded species rather than from ligand-free and fully saturated native species as is commonly believed. (With A. Shrake).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36105-10 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Influences of Macromolecular Crowding on Biochemical Systems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

S. B. Zimmerman, Ph.D. Research Chemist LMB/NIDDKD

L. D. Murphy, Biologist LMB/NIDDKD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL STAFF YEARS

1.9

PROFESSIONAL:

1.9

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The high concentrations of macromolecules within cells result in large excluded volumes, potentially causing very large shifts in rates and equilibria of reactions involving other macromolecules. Prior studies of such macromolecular crowding effects on DNA by ourselves and others have been restricted to empirical studies of model systems, leaving unanswered basic questions about DNA function under crowded conditions. We have addressed two of those questions.

First we have studied the effective volume of DNA and related materials in crowding interactions. The two-phase distribution assay for volume occupancy described earlier was used to determine the effective volumes in crowding interactions of a series of small double- and single-stranded pieces of DNA. Their behavior is fully consistent with a simple theoretical treatment, the available volume model, used for predicting excluded volume effects. These results are "in press" in Biopolymers.

Second, we are currently measuring the crowding effects of actual cellular material, cytoplasmic extracts from E. coli, on a DNA test system. The test system measures the rate of in vitro cohesion of restriction fragments of λ DNA. Preliminary results indicate accelerations of at least 1-2 orders of magnitude.

In addition to these experimental studies, I am writing a review on macromolecular crowding in collaboration with Allen Minton (also of NIDDKD) for the 1992 edition of Annual Reviews of Biophysics and Biophysical Structure.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01DK36106-04LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (60 characters or less. Title must fit on one line between the borders.)

Developmental Regulation of Differential Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. (Name, title, laboratory, and institute affiliation.)

PI: Alan Wolfe, Visiting Scientist

COOPERATING UNITS (if any)

LAB BRANCH

Laboratory of Molecular Biology

SECTION Section on Physical Chemistry

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL STAFF YEARS

0

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unstructured type. Do not exceed the space provided.)

Terminated

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36108-05 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Mechanism of Retroviral DNA Integration

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

Robert Craigie, Ph.D.

Visiting Scientist

LMB/NIDDK

Kiyoshi Mizuuchi, Ph.D.

Chief, Genetic Mechanisms

LMB/NIDDK

Frederic Bushman, Ph.D.

Special Volunteer

LMB/NIDDK

Alan Engelman, Ph.D.

IRTA

LMB/NIDDK

Myung Soo Lee, Ph.D.

Special Volunteer

LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Genetic Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, Maryland 20892

TOTAL STAFF YEARS:

4

PROFESSIONAL:

4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Integration of a DNA copy of the retroviral genome into a chromosome of the host cell is an essential step in the retroviral replication cycle. The objectives of this project are to understand the detailed molecular mechanism of the integration reaction and to facilitate the development of inhibitors that block this step in the replication cycle.

We have previously shown that the viral integrase protein carries out the central steps of the integration reaction in vitro. Our recent work has continued to focus on the biochemical activities of the HIV integrase protein. Integrase catalyzes two distinct reactions: site-specific cleavage of two nucleotides from the 3' ends of the viral DNA and a subsequent reaction that inserts the resulting processed ends into a target DNA. Stereochemical analysis of these reactions supports the view that they both proceed by a one-step mechanism, not involving a covalent intermediate between integrase and the DNA substrate. We have analyzed the functional organization of HIV integrase by expressing and purifying mutant proteins with changes at selected amino acid positions, or deletions extending from the N- or C-terminus. Substitution of conserved amino acids in a central part of the protein that is highly conserved among retroviral integrases abolished catalytic activity, suggesting a key role for this part of the protein in catalysis. In contrast, a conserved motif near the N-terminus is not essential for catalysis, but may be important for protein-DNA or protein-protein interactions.

Retroviral DNA made by reverse transcription after infection of a sensitive cell exists as part of a large nucleoprotein complex, derived from the viral core. Although purified integrase protein carries out the DNA cutting and joining steps of integration in vitro, some aspects of the reaction are not efficiently reproduced with integrase alone, but are reproduced when in vitro reactions are carried out with complexes isolated from infected cells. We are analyzing such complexes, isolated from cells infected with Moloney murine leukemia virus, to determine the factors that contribute to this greater fidelity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36109-5 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Aids Related Proteins: Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

David R. Davies, Ph.D. Chief, Sec. on Molecular Structure LMB/NIDDK

Alison B. Hickman, Ph.D. IRTA LMB/NIDDK

Enid S. Silverton, Ph.D. Research Chemist LMB/NIDDK

Sun Daopin, Visiting Associate LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The crystal structure of Transforming Growth Factor Beta II has been determined.

The HIV integration protein has been the subject of isolation and purification procedures to establish methods for the preparation of adequate protein for crystallization trials. Solubility problems associated with the original HIV I preparation have led to a search for related proteins that are more suitable for crystallization.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36110-03 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Control of Gene Expression During Chicken Erythrocyte Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

Joanne Nickol, Ph.D. Research Chemist LMB/NIDDK

COOPERATING UNITS (if any)

Lab of Molecular Carcinogenesis, NCI, NIH (M. Cripps, M. Bustin)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

.50

PROFESSIONAL:

.50

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Project Terminated

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36111-03 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural Molecular Biology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

Joanne Nickol, Ph.D. Research Chemist LMB/NIDDK

COOPERATING UNITS (if any)

Lab of Biochemical Metabolism, NIDDK, NIH (D.C. Rau)
University of Calgary Medical School, Calgary, Alberta, Canada D. Bazett-Jones)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD, 20892

TOTAL STAFF YEARS:

.25

PROFESSIONAL:

.25

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Project Terminated

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36113-2 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Channeling in Biosynthesis of Histidine

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

Robert G. Martin, Ph.D. Chief Section on Microbial Genetics LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL STAFF YEARS

.50

PROFESSIONAL

.50

OTHER

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Project Terminated

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36114-2 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Structural Studies of Molecular Recognition

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute)

Eduardo A. Padlan, Ph.D. Visiting Scientist LMB/NIDDK

Chantal Abergel, Ph.D. Visiting Fellow LMB/NIDDK

Jennifer P. Tipper, Ph.D. Temporary GS-12 LMB/NIDDK
(Dr. Tipper joined the group in March 1992)

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

X-ray diffraction data have been collected from crystals of the Fab of CC49, a murine monoclonal antibody against solid adenocarcinoma, and molecular replacement analysis of the crystal structure is in progress.

The known structures of Fabs have been analyzed to determine which framework residues need to be preserved in the 'humanization' of xenogeneic antibodies by CDR-grafting.

A model of the extracellular portion of the alpha-subunit of the human high-affinity IgE receptor has been built.

The binding of various viral and self peptides to the murine class I MHC antigen, H-2Ld, has been modelled.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36115-2 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of the Potential Use of Catalytic Antibodies Against AIDS

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

Eduardo A. Padlan, Ph.D. Visiting Scientist LMB/NIDDK

COOPERATING UNITS (if any)

Ettore Appella, M.D. Medical Officer LCB/NCI Birgit A. Helm, Ph.D. Lecturer
Thomas J. Kindt, Ph.D. Chief LIG/NIAID Univ. of Sheffield

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1

PROFESSIONAL:

1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Three peptidic transition-state analogs have been proposed from highly-conserved polypeptide segments of the HIV-1 envelope glycoprotein and two of these analogs have been synthesized and used as immunogens for the production of murine monoclonal antibodies. Several hundred hybridomas have been shown to bind to the transition-state analog used for immunization and several of these show differential binding to the transition-state analog vs the original-sequence peptide.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36116-1 LMB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

E. coli Genes Whose Expression is Altered by Salicyl Alcohol

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

Robert G. Martin, Ph.D. Chief Section on Microbial Genetics LMB/NIDDK

COOPERATING UNITS (if any)

J.L. Rosner, Ph.D. Research Biologist LMB/NIDDK

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

.50

PROFESSIONAL:

.50

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Salicylates, like heat shock and UV damage, induce a panoply of changes in E.coli including (but not limited to): the induction of resistance to certain classes of antibiotics and sensitivity to others; alteration in outer membrane protein composition; and increase sensitivity to Cd⁺⁺. Some of these effects may be of the weak acid variety, but the majority are not as they are also induced by salicyl alcohol. In an attempt to dissect and define the action of salicyl alcohol on E. coli, we have constructed two libraries of 10⁵ independent transpositional events, each containing the lac Z gene (with and without translational initiation signals) inserted at random about the E. coli genome. We are currently in the process of screening these libraries for transpositional events in which the lac Z gene is induced or repressed by salicyl alcohol and in which the resistance of nalidixic acid is not increased by salicyl alcohol. A number of potential mutants have been identified and we are beginning to map and characterize them.

Annual Report of the Clinical Endocrinology Branch
National Institute of Diabetes and
Digestive and Kidney Diseases

This branch has been reorganized as part of the Genetics and Biochemistry Branch.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK #5000-25

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thyroid Hormone Interactions with Cells and Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

J. Robbins	Chief, Endocrinology Section,	GBB, NIDDK
Luigi Bartalena	Visiting Scientist	GBB, NIDDK
Marcia Phyllaeier	Biologist	GBB, NIDDK

COOPERATING UNITS (if any)

Salvatore Benvenaga (University of Messina, Italy)

Daniel Rader (NHLBI)

Mark Lakshmanan, Case Western Reserve University

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Transferred to Project #Z01 DK 52016-01 GBB

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK45009-25															
PERIOD COVERED October 1, 1991 to September 30, 1992																	
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Studies of Thyroid Diseases																	
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;"> PI: Jacob Robbins </td> <td style="width: 33%; vertical-align: top;"> Chief, Endocrinology Section </td> <td style="width: 33%; vertical-align: top;"> GBB, NIDDK </td> </tr> <tr> <td colspan="3" style="height: 10px;"></td> </tr> <tr> <td style="vertical-align: top;"> Others: Taisheng Lee </td> <td style="vertical-align: top;"> Clinical Associate </td> <td style="vertical-align: top;"> GBB, NIDDK </td> </tr> <tr> <td style="vertical-align: top;"> Desiree Pineda </td> <td style="vertical-align: top;"> Clinical Associate </td> <td style="vertical-align: top;"> GBB, NIDDK </td> </tr> <tr> <td style="vertical-align: top;"> M. Phyllaier </td> <td style="vertical-align: top;"> Biologist </td> <td style="vertical-align: top;"> GBB, NIDDK </td> </tr> </table>			PI: Jacob Robbins	Chief, Endocrinology Section	GBB, NIDDK				Others: Taisheng Lee	Clinical Associate	GBB, NIDDK	Desiree Pineda	Clinical Associate	GBB, NIDDK	M. Phyllaier	Biologist	GBB, NIDDK
PI: Jacob Robbins	Chief, Endocrinology Section	GBB, NIDDK															
Others: Taisheng Lee	Clinical Associate	GBB, NIDDK															
Desiree Pineda	Clinical Associate	GBB, NIDDK															
M. Phyllaier	Biologist	GBB, NIDDK															
COOPERATING UNITS (if any) J. Norton, Surgery Branch, NCI; J. Reynolds, Nuclear Medicine, CC; M. Merino, Laboratory of Pathology, NCI; C. Meyers, NCI																	
LAB/BRANCH Genetics and Biochemistry Branch																	
SECTION Endocrinology Section																	
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892																	
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:															
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div> <input type="checkbox"/> (b) Human tissues </div> <div> <input type="checkbox"/> (c) Neither </div> </div>																	
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <div style="height: 300px; border: 1px solid black;"></div>																	

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01DK45014-20 CEB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
 Membranes and Secretion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. Name, title, laboratory, and institute affiliation)

PI: J. Wolff	Associate Chief	CEB, NIDDK
Others: D. Sackett	Expert	CEB, NIDDK
L. Knipling	Technician	CEB, NIDDK

COOPERATING UNITS (if any)

T. Shiver, NICHD

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

This project has been transferred to Z01 DK 24941 LBP.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK45016 CEB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thyroid Hormone Secretion and the Function of Microtubules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Wolff	Associate Chief	CEB, NIDDK
Others:	D. Sackett	Senior Staff Fellow	CEB, NIDDK
	L. Knipling	Technician	CEB, NIDDK
	G. Obi	Guest Worker	CEB, NIDDK
	M. Bifulco	Guest Worker	CEB, NIDDK
	C. Laezza	Guest Worker	CEB, NIDDK

COOPERATING UNITS (if any)

Jay Knutsen, NHLBI
 J. Thompson, NIDR

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreserved type. Do not exceed the space provided.)

Project has been transferred to Z01-DK 23900 LBP.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 45018 CEB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Adenylyate Cyclase and Other Extracellular products of B. Pertussis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator (Name, title, laboratory, and institute affiliation))

PI: J. Wolff	Associate Chief	CEB, NIDDK
Others : D. Sackett	Expert	CEB, NIDDK
L. Knipling	Technician	CEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

This project is inactive and has been transferred to Z01-DK24942 LBP.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 45020-16

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthesis of Thyroxine Transport Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Robbins

Chief, Endocrinology Section

GBB, NIDDK

Others: L. Bartalena
M. Phyllaier

Visiting Associate
Biologist

GBB, NIDDK
GBB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
☐ (a1) Minors
☐ (a2) Interviews

☐ (b) Human tissues

☐ (c) Neither

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been discontinued and incorporated into Project No. Z01-DK-52016-01 GBB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 45028-14

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thyroid Hormone-Cell Interactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Robbins

Chief, Endocrinology Section

GBB, NIDDK

Others:

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been discontinued and incorporated into Project No. Z01-DK-52016-01 GBB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 45033-08

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mapping of Triiodothyronine Responsive Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. Nikodem

Senior Investigator

GBB, NIDDK

Others: J. Lazar

Visiting Fellow

GBB, NIDDK

B. Desvergne

Visiting Associate

GBB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Mechanisms of Gene Regulation

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Transferred to Project #Z01 DK 52021-01 GBB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 45034 CEB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Specific Rat Liver mRNAs by Thyroid Hormone

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. Nikodem CEB, NIDDK

Others: R. Lippoldt CEB, NIDDK
 J.E. Rall CEB, NIDDK
 M.K. Song CEB, NIDDK
 D. Grieco CEB, NIDDK

COOPERATING UNITS (if any)

Dr. S.M. Aloj and Dr. L. Kohn, LBM, NIDDK

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unenriched type. Do not exceed the space provided.)

This project has been transferred to Z01-DK-52022 GBB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 45038-04

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Biology of Thyroid Hormone Receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. Nikodem Senior Investigator GBB, NIDDK

Others: E. Jannini	Guest Worker	GBB, NIDDK
P. Hallenbeck	Staff Fellow	GBB, NIDDK
R. Lippoldt	Chemist	GBB, NIDDK
M. Phyllaier	Technician	GBB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH Genetics and Biochemistry Branch

SECTION Mechanisms of Gene Regulation

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Transferred to Project #Z01 DK 52019-01 GBB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 45040-03

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of Thyroid Hormone on Synthesis of Myelin Basic Protein

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V. Nikodem Senior Investigator GBB, NIDDK

Others: A. Farsetti	Visiting Fellow	GBB, NIDDK
F. Bogazzi	Visiting Fellow	GBB, NIDDK
D. Pineda	Clinical Associate	GBB, NIDDK
B. Dozin-Quarto	Visiting Scientist	GBB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Mechanisms of Gene Regulation

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Transferred to Project #Z01 DK 52018-01 GBB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 45041-02
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT <small>(80 characters or less. Title must fit on one line between the borders.)</small> Regulation of Anteroposterior Patterning in Early Frog Development		
PRINCIPAL INVESTIGATOR <small>(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)</small> <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> PI: S. Sato </div> <div style="width: 35%;"> Senior Staff Fellow </div> <div style="width: 30%;"> GBB, NIDDK </div> </div>		
<div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> Others: V. Agarwal S. Witta </div> <div style="width: 35%;"> Visiting Associate Visiting Fellow </div> <div style="width: 30%;"> GBB, NIDDK GBB, NIDDK </div> </div>		
COOPERATING UNITS <small>(if any)</small> Dr. William Hayes, LDN, NICHD		
LAB/BRANCH Genetics and Biochemistry Branch		
SECTION Mechanisms of Gene Regulation		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div style="width: 30%;"> <input type="checkbox"/> (b) Human tissues </div> <div style="width: 30%;"> <input type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK <small>(Use standard unrounded type. Do not exceed the space provided.)</small> Transferred to Project Number Z01 DK 52020-01 GBB.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01DK45042-02CEB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (100 characters or less. Title must fit on one line between the borders.)

The Role of Xenopus-posterior (Xpo) in Early Frog Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: S. Sato Senior Staff Fellow

COOPERATING UNITS (if any)

Dr. Tomsargent, Laboratory of Molecular Genetics, NICHD
Dr. Christine Holt, Department of biology UCSD

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and Action section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL STAFF YEARS

0

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES.

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unprocessed type. Do not exceed the space provided.)

This project has been terminated.

ANNUAL REPORT OF THE METABOLIC DISEASES BRANCH
National Institute of Diabetes and
Digestive and Kidney Diseases

The general goals of the Branch are to investigate the mechanism of action of hormones controlling ion transport and mineral metabolism and to investigate the immunological and pathological factors mediating kidney disorders. The branch currently includes sections of Mineral Metabolism (Dr. Marx), Endocrine Regulation (Acting Chief, Dr. Spiegel), Renal Cell Biology (Dr. Balow). Integration of these sections is related to common interests in the pathophysiology of metabolic disorders which interface with the kidney. Systems under study include renal and skeletal tissue, transgenic mice, isolated cells (kidney and parathyroid in culture), hormone receptors (beta adrenergic, parathyroid hormone, calcitonin and 1,25-dihydroxy-vitamin D), parathyroid cell growth factors, and T cell and B cell function in disorders of immunoregulation.

Analysis of Hormone Receptor

Interactions with several hormone receptors regulating growth or adenylate cyclase are under study. Specific receptors have now been identified on turkey erythrocytes, parathyroid cells, pineal cells, rat, guinea pig and monkey lung membrane preparations, bovine parathyroid endothelial cells, rat osteosarcoma cells and rat liver membranes. Control of receptor biosynthesis in isolated cell culture systems is being studied with a view toward gaining knowledge about the molecular biology of receptors and how they are linked to intracellular response systems. A new method has been developed for immunocytology of cAMP and cGMP to study compartmentalization of signal transduction.

Primary Hyperparathyroidism and Familial Hypercalcemia

Clinical studies are continuing on primary hyperparathyroidism and its familial variants. Detailed family screening and case findings have produced approximately 85 kindreds for analysis. These studies allowed segregation of the most common familial variants into two distinct disease syndromes - familial multiple endocrine neoplasia type 1 (FMEN I) and familial hypocalciuric hypercalcemia (FHH). FHH was distinguished from FMEN I by 1) virtually a 100% penetrance for hypercalcemia before age 20, 2) milder clinical manifestations - low incidence of recurrent nephrolithiasis or recurrent peptic ulceration, 3) no hypercalciuria, 4) normal basal concentrations of gastrin, and 5) poor response to subtotal parathyroidectomy. Distinction between the two syndromes, both inherited as

autosomal dominant traits, is important because in FHH the clinical course is generally milder and subtotal parathyroidectomy is less likely to be beneficial. In several FHH kindreds one or more members have exhibited life-threatening primary hyperparathyroidism in the neonatal period. This may result sometimes from a double dose of the FHH gene. Dispersed parathyroid cells from one severely affected neonate showed a striking decrease in sensitivity of PTH secretion to extracellular calcium. This disorder may reflect mutation in a gene that directs calcium recognition in both the parathyroid and renal tubular cell

Familial multiple endocrine neoplasia type I (FMEN1) is an autosomal dominant disorder characterized by hyperfunction of parathyroids, pancreatic islets, and anterior pituitary. Affected organs show features suggestive of increased proliferation. Virtually all subjects expressing the gene show primary hyperparathyroidism. Primary hyperparathyroidism is usually first recognizable between ages 20-40, and it shows a high recurrence rate after subtotal parathyroidectomy (approximately 50% after 10 years). We have evaluated multiple indices for use in screening in a very large kindred. We tested 221 members and newly identified 16 as carriers. Albumin-adjusted calcium and PTH were most useful; gastrin and prolactin analyses were not useful for screening but showed promise in followup of known carriers. Analysis in this family has revealed linkage to a locus on the long arm of chromosome 11. The MEN1 gene is a growth suppressor gene like the retinoblastoma gene; MEN1 related tumors are being screened for loss of heterozygosity at this locus. Such loss of heterozygosity has established that most parathyroid tumors in FMEN1 are monoclonal. Tumors with small deletions could speed identification of the MEN1 gene. Similar studies suggest that inactivation of the MEN1 gene also contributes to neoplasia in over 25% of sporadic parathyroid adenomas. [Drs. Friedman, DeMarco, A. Bale, Brandi, Norton, Spiegel, Aurbach, Marx]

With cultured bovine parathyroid cells, we found abnormally high mitogenic activity in plasma from 23 of 27 subjects with FMEN1. Well-characterized growth factors or known parathyroid secretagogues showed far less parathyroid mitogenic activity than these FMEN1 plasmas. The mitogenic factor(s) appears to be a protein of 14,000 mw. We have begun purifying this factor for further characterization. We have obtained evidence that the factor is related to basic fibroblast growth factor. [Drs. Zimring, Brandi, Sakaguchi, Aurbach, Marx].

Studies on noninvasive and invasive modes of localizing parathyroid tumors continue. Parathyroid adenoma localization has been evaluated using the new non-invasive magnetic resonance imaging technique. Initial results were disappointing but the acquisition of a specialized neck collar has led to better resolution in the paratracheal and mediastinal areas. Patients are currently under evaluation with this new technique. A high

degree of success has been obtained in localizing tumors through vascular catheterization procedures. Parathyroid arteriography developed and performed by Dr. John Doppman afforded, in approximately 450 of cases tested, the identification of abnormal masses of tissue proven at surgery to be parathyroid. In the most difficult cases, localization of parathyroid tissue can be aided by identifying high concentrations of parathyroid hormone by radioimmunoassay in veins draining the lesion. Fine needle aspiration is another new method that can obviate other invasive localization procedures. We have aspirated with guidance by computerized tomography or ultrasound approximately 20 such lesions that were subsequently confirmed surgically as parathyroid. RIA of the aspirates showed high concentrations of PTH in all but one. Eight mediastinal adenomas have been treated nonsurgically by percutaneous injection via catheter of occlusive agents into the arterial blood supply with 7 complete and one partial remissions. [Drs. Aurbach, Marx, Spiegel, Weinstein, NIDDK: Dr. Norton, Fraker and Alexander NCI, Drs. Doppman, Miller, and others, Diagnostic Radiology, CC].

Secretion of Parathyroid Hormone

PTH secretion from parathyroid glands in vivo and cells in vitro is controlled by intracellular calcium and cyclic AMP. Control by calcium is altered in certain pathologic states (glandular adenomas, carcinomas and perhaps hyperplasia). Agents that alter cellular cAMP change PTH secretion in the same direction. Calcium decreases cellular cAMP, but most of its effect to inhibit secretion is independent of changes in cellular cAMP.

Calcium inhibition of parathyroid hormone secretion is controlled through a complex set of mechanisms. We have shown previously that classical voltage-sensitive calcium channels are important in controlling parathyroid hormone secretion and that their action is mediated through a guanine nucleotide regulatory protein. It is also known that the growth of parathyroid cells is controlled by calcium. We have utilized a cloned rat parathyroid cell line (PT-r) to study these phenomena.

Parathyroid hormone synthesis and secretion is well characterized to be stimulated by low calcium. However, there are conflicting data about parathyroid cell growth regulation by calcium. The conflict may have derived from the differences in system and some variables in each system. PT-r cells are clonal and have been proven to show negative regulation of growth by calcium. Previously, we reported that PT-r cells bear two high affinity receptors for acidic FGF (aFGF), and that at least a subpopulation of the receptors with a higher molecular mass carries heparan sulfate (HS) Glycosaminoglycan chains which give the receptor higher affinity. Now I have found that the parathyroid cell express aFGF, and that aFGF receptors with lower affinity apparently translocate in response to changing

extracellular calcium concentrations. Expression of both aFGF mRNA and peptide is suppressed by calcium. Cells have more ligand-accessible receptors on the cell surface at lower calcium concentrations. The apparent translocation of receptors is temperature-dependent but independent of de novo protein synthesis. In concordance with the apparent translocation of aFGF receptors, thymidine incorporation is stimulated by decreasing extracellular calcium concentrations with further stimulation by aFGF. Anti-aFGF antibody inhibits thymidine incorporation by 15% at 0.7 mM Ca^{2+} and by more than 30% in the cells exposed to low calcium shortly before adding [^3H]thymidine. An aFGF autocrine system including the apparent translocation of aFGF receptors may explain the mechanism through which parathyroid cell growth is regulated by calcium. [Dr. Sakaguchi]

Vitamin D Resistance and Related Disorders

The role of $1,25(\text{OH})_2\text{D}_3$, the most potent natural metabolite of vitamin D, has been assessed in hypocalcemic states. This very rapidly acting drug has simplified the management of hypocalcemia following parathyroidectomy: during this time skeletal remineralization imposes large but rapidly diminishing requirements for calcium.

We have evaluated patients with extreme resistance to $1,25(\text{OH})_2\text{D}$. This can be a transient state as following parathyroidectomy or a permanent state as in familial cases. We have evaluated 20 patients with familial resistance to $1,25(\text{OH})_2\text{D}$. Most patients have hypocalcemic rickets, usually with associated total alopecia. The alopecia is associated with the highest grades of resistance to $1,25(\text{OH})_2\text{D}$, implicating calcitriol in physiology of the hair follicle. Mineral homeostasis is usually improved by treatments that sustain $1,25(\text{OH})_2\text{D}$ levels at 10-100 times normal. Intestinal response to $1,25(\text{OH})_2\text{D}$ can be documented repeatedly with a new stable isotope technique [Drs. Yergey, Viera, Marx].

Specific intracellular defects have been evaluated using cultured skin fibroblasts from these patients. With skin fibroblasts cultured from normals, properties of the $1,25(\text{OH})_2\text{D}$ -receptor can be identified by binding in soluble extracts, by nuclear uptake of hormone with intact cells, or by elution of occupied receptor from DNA-cellulose. Fibroblasts from patients with familial resistance to $1,25(\text{OH})_2$ have shown a spectrum of defects including nonfunctional receptors, diminished numbers of receptors, and receptors with decreased hormone binding affinity. Among cases with normal hormone binding sites on the receptors some show receptors with deficient binding to nucleus while others show normal binding to nucleus but abnormal interaction with nonspecific DNA (as DNA-cellulose). Cellular action of $1,25(\text{OH})_2\text{D}_3$ can be analyzed by measuring its induction of the $25(\text{OH})\text{D}$ 24-hydroxylase enzyme system. Cultured skin fibroblasts from all patients with hereditary resistance to $1,25(\text{OH})_2\text{D}$ exhibit defects in this induction. Immunocytology reveals

multiple rapid steps of reorganization of vitamin D receptors after calcitriol addition. Specific disruptions in these steps can be imaged in mutant cells from patients. Four different homozygous, point mutations in the gene for the Vitamin D receptor have so far been identified as the cause of this disorder in six kindreds. [Drs. Marx, Barsony, MDB, NIDDK; Dr. Liberman, Israel; Drs. Pike (Baylor) and DeLuca (Madison)].

Fibroblast lines from patients with hereditary extreme resistance to $1,25(\text{OH})_2\text{D}_3$ are being used to probe for normal functions of the $1,25(\text{OH})_2\text{D}_3$ receptor. We have shown that $1,25(\text{OH})_2\text{D}_3$ can elevate intracellular cyclic GMP very rapidly (within 1-3 minutes). This response showed affinity and analog specificity characteristic of the $1,25(\text{OH})_2\text{D}_3$ receptor and was absent in all "mutant" fibroblast lines although they retained a rapid cGMP response to nitroprusside and to androgens. Thus a $1,25(\text{OH})_2\text{D}_3$ receptor mediates this rapid response. Immunocytology revealed that, after $1,25(\text{OH})_2\text{D}_3$ addition, cGMP accumulates rapidly about the reorganizing vitamin D receptors [Drs. Barsony, Marx].

KIDNEY DISEASE SECTION

The Kidney Disease Section conducts parallel clinical and laboratory research centered on human glomerular diseases, experimental models of immune-mediated renal disorders, mechanisms of immunosuppression, and normal biology of various types of kidney cells. Patient and animal tissues are used to study pathogenetic mechanisms, primarily involving immune functions, cytokines and growth factors. The effects of various immunosuppressive drugs at the level of gene regulation and transcription and novel immunosuppressive drug therapies which might have salutary effects on the course of lupus nephritis and membranous nephropathy are under study.

Immunopathogenesis of glomerulonephritis.

Murine models are being utilized to investigate the different components of lupus nephritis. The modulating effects of cyclophosphamide on immune responses in normal mice and on the renal lesions of nephritic mice are being investigated. Studies of differences among the murine strains have provided new approaches to study of the diverse manifestations and response to treatment of human lupus nephritis. (Austin, Patel, Balow).

Regulation of lymphocyte gene expression.

Dysregulated cell mediated immune responses are present in subjects with most forms of glomerulonephritis. Studies of the mechanisms of control of B and T cell activation, including regulation of immunoglobulin and cytokine genes by nuclear factors are being pursued. Special emphasis is placed on studies of the effects of various immunoregulatory agents, such as corticosteroids, cyclosporine, and cyclophosphamide. (Boumpas, Paliogianni, Ahuja, Balow).

Proliferative lupus nephritis.

Previous studies have shown that intermittent pulse cyclophosphamide therapy is superior to conventional prednisone in management of lupus nephritis, but no direct comparisons of pulse corticosteroids and pulse cyclophosphamide have been performed. In a recently completed and analyzed long-term clinical trial, patients with proliferative lupus nephritis were treated with pulse methylprednisolone or pulse cyclophosphamide to compare these two types of drugs and to assess whether intensity or duration of cyclophosphamide therapy is more important in stabilizing the renal disease.

In patients with severe lupus nephritis manifested by renal function impairment and or highly active renal pathology an extended course of pulse cyclophosphamide was significantly more effective than pulse methylprednisolone in reducing the risk of progressive renal failure; moreover, a regimen of monthly pulse

cyclophosphamide over 6 months, followed by quarterly maintenance treatments significantly reduced the probability of major exacerbations of lupus, compared to a 6 month course of pulse cyclophosphamide alone. Thus, evidence from this controlled trial indicates that intermittent pulse methylprednisolone does not match pulse cyclophosphamide in the management of severe proliferative lupus nephritis.

This work on lupus nephritis has attracted national and international attention. Dr. Balow has been invited to present this work at the American Society of Nephrology in November 1991, to deliver a lecture on the subject to the British Renal Association in April 1992, and to lead a symposium on treatment of lupus nephritis at the Canadian Society of Nephrology in September 1992.

Immunologic studies of changes in lymphoid cell function by the various drug regimens are being pursued to identify techniques which will maximize efficacy and to improve monitoring of drug treatment. (Balow, Austin, Boumpas, MacKay).

Membranous nephropathy.

Membranous nephropathy is associated with substantial cardiovascular morbidity from nephrotic syndrome and causes an insidious loss of renal function in patients with lupus and in those patients with idiopathic forms of this disease. Preliminary evidence indicates that the immunopathogenesis of membranous nephropathy is distinct from that of most proliferative forms of glomerulonephritis. Current protocols involve examination of the pathophysiology and histopathology of the glomerular lesions in membranous nephropathy, as well as evaluation of the comparative efficacy of prednisone, cyclophosphamide and cyclosporin A in patients idiopathic and lupus related forms of this renal disease. (Balow, Austin, MacKay).

Transforming growth factor and glomerular reactions.

The mechanisms responsible for normal growth and for pathogenic cellular reactions within the glomerulus are poorly understood. The signal transducing agent, transforming growth factor-beta (TGF- β), has a complex interaction with glomerular cells. Studies are underway to characterize the nature of the receptors for this growth factor on glomerular cells. Several parameters, such as proliferation, fibronectin secretion and proteoglycan synthesis, will be used to evaluate the responses of these cells to binding of TGF- β . Studies of factors which regulate TGF- β receptors are underway. Information learned from these experiments should facilitate studies of the role of the TGF- β ligand and its receptors in experimental models of glomerulonephritis. (MacKay).

RENAL CELL BIOLOGY SECTION
Studies of the pathogenesis of glomerulosclerosis

The renal cell biology section is interested in the cellular and molecular mechanisms leading to glomerular scarring, the emphasis being on non-immune diseases. The kidney disease of diabetes mellitus is the major disease studied. Our hypothesis is that glomerulosclerosis results from abnormalities in the rate of proliferation and matrix turnover by resident glomerular cells. This is being investigated in vitro, using clonal lines of glomerular cells, and in vivo using transgenic mice. We have developed a new technique to quantitatively study the synthesis and degradation of glomerular matrix components in vivo. The method consists of microdissection of single mouse and human glomeruli, reverse transcription in situ. This was followed by the polymerase-chain reaction (PCR). We have developed a method to quantitate PCR, so that we can examine the relative amounts of the different mRNAs coding for the various basement membrane collagens and the enzymes that degrade collagens. This technique is now being applied to extracellular matrix turnover in glomeruli of mice transgenic for growth hormone and to human glomeruli obtained from nephrectomy specimens. Current investigators are: L. Striker, G. Striker, T. Doi, E. Peten, S. Elliot, M. Carome, C. Pesce, K. Schmidt, and C. Yang.

I. Glomerulosclerosis

A. In Vivo Studies.

1. Mice transgenic for GH: We found that mice transgenic for GH develop severe glomerulosclerosis leading to kidney failure, and that they had a disproportionate increase in the size of the glomeruli. Thus, GH may have effects in addition to those on cell number. We found an increase in mRNAs coding for collagen types I and type IV in glomeruli, using a method consisting of microdissecting mouse glomeruli followed by a quantitative analysis of polymerase chain reaction products. We are now investigating mice transgenic for mutated GH species in which body size and glomerulosclerosis are regulated in an independent manner. Study of the glomerular changes in these mice may lead to further understanding of the pathogenesis of glomerulosclerosis (L. Striker, E. Peten, D. Yang, G. Striker)

2. Nonobese diabetic (NOD) mice: NOD mice develop autoimmune diabetes mellitus. We compared glomerular size, morphology, composition of the sclerotic extracellular matrix, and urine protein excretion rate in mice before and after the onset of diabetes mellitus. An increase in glomerular size, mesangial sclerosis, and proteinuria rapidly followed the onset of hyperglycemia. We are synchronizing the onset of clinical diabetes mellitus with streptozotocin injections and assessing matrix synthesis in isolated glomeruli, and total kidney. These mice could provide a good model of nephropathy in a genetically determined model of type I diabetes mellitus. (T. Doi, D. Yang, L. Striker, G. Striker)

3. Expression of genes coding for collagens and degradative enzymes in Human Glomeruli

We have begun to measure gene expression of several genes involved in glomerulosclerosis in human microdissected reverse-transcribed glomeruli, using competitive PCR. In patients with and without glomerulosclerosis, we have begun comparisons of mRNAs coding for 1) the $\alpha 1$, $\alpha 2$, $\alpha 3$ chains of type IV collagen; and 2) The tissue inhibitors of metalloproteinases, TIMP I and TIMP II. These species are all present in higher amounts in sclerotic, compared to normal glomeruli. This method provides the first quantitative means to study the amounts of types of molecules responsible for the molecular events leading to glomerulosclerosis in man. (L. Striker, G. Striker, E. Peten, M. Carome, D. Yang, S. Elliot).

4. Kidney disease in diabetic Pima Indians. We have undertaken a retrospective study of the glomerular lesions in diabetic Pima Indians. We developed morphometric methods to measure glomerular size to determine whether glomerulosclerosis is associated with hypertrophy. We found that Pima Indians have large glomeruli, and that glomerular size in Pimas with diabetes mellitus did not differ from non-diabetics. The size of glomeruli in African Americans and Caucasians was also determined, utilizing forensic autopsies. Glomerular size paralleled the incidence of endstage kidney disease in these populations, i.e., Pima>African Americans>Caucasians. (L. Striker, P. Bennett, G. Striker, C. Pesce, K. Schmidt)

5. Ablation model. Subtotal nephrectomy in rats is a well-studied model of progressive glomerulosclerosis. Analyzing the early cellular events that may lead to glomerular scarring, we found an increase in glomerular cell turnover which occurred weeks before there was an increase in extracellular matrix deposition. (L. Striker, C. Pesce, G. Striker)

B. In Vitro Studies.

1. Murine and human glomerular cells: We have developed lines of mouse epithelial, mesangial, and endothelial cells from normal mice and from several strains of transgenic mice and have been investigating their response to growth peptides and advanced glycosylation endproducts. Mesangial cells synthesize collagen types IV and I as well as metalloproteinases I and their inhibitor (TIMP I). When these cells are exposed to advanced glycosylation endproducts, there is a rapid increase in collagen synthesis, providing evidence that these products may be one cause of glomerulosclerosis in diabetes mellitus. (T. Doi, S. Elliot, M. Carome, E. Peten, L. Striker, G. Striker)

2. Analysis of lines of mesangial cells from mice that develop glomerulosclerosis: We developed mesangial cell lines from NOD mice and from mice transgenic for bovine growth hormone. We are examining their cell cycle and synthesis of extracellular matrix. We found phenotypic changes, and postulated that one cause of the glomerulosclerosis was related to this change. (L. Striker, T. Doi, E. Peten, M. Carome, S. Elliot, G. Striker)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 43002-27 MD
PERIOD COVERED October w, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) <u>Structure, Secretion and Mechanism of Action of Parathyroid Hormone</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	G.D. Aurbach, M.D. Chief	MDB, NIDDK
OTHERS:	S. Doi, M.D. Visiting Associate	MDB, NIDDK
	K. Sakaguchi, M.D., PhD Visiting Associate	MDB, NIDDK
	D. Coleman, Ph.D. IRTA	MDB, NIDDK
	Y. Takagi, M.D. Visiting Fellow	MDB, NIDDK
COOPERATING UNITS (if any) Endocrine Unit, Massachusetts General Hospital National Institute of Dental Research, BRB		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Endocrine Regulations Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.5	3.5	1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> It is the purpose of this project to study the secretion, function, and mechanism of action of parathyroid hormone, its relationship to human disease, and to develop clinically useful tests for circulating parathyroid hormone. From these studies it is expected that one can understand the pathophysiology of certain metabolic diseases of bone and endocrine disturbances. The entire structures of bovine, porcine, rat and human parathyroid hormone have been determined. Synthetic polypeptides representing bovine rat and human <u>parathyroid hormone</u> have been synthesized. These molecules show all the biological properties of the native hormonal polypeptides. Highly sensitive radioimmunoassays for the hormone have been developed and are being modified further for improved clinical diagnostic parameters. Studies show that the mechanism of action of the hormone is mediated through direct hormonal activation of <u>adenylate cyclase</u> in <u>bone</u> and <u>kidney</u>. Isolated parathyroid cells and culture systems have been developed that allow studies on secretory control of <u>parathyroid hormone</u>, and provide test systems to elucidate the pathophysiology of certain hypoparathyroid and hyperparathyroid states. </p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 43003-27 MD
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders) Studies on the Mode of Action of Thyrocalcitonin		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sect. MDB, NIDDK
OTHERS:	J. Barsony, M.D.	Visiting Scientist MDB, NIDDK
COOPERATING UNITS (if any) NONE		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Mineral Metabolism Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.1	0.1	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided) <p> The purpose is to study the interaction of <u>calcitonin</u> with its specific receptor target organs. The current investigations should provide further insight into the structure-function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful for characterizing hormone <u>receptors</u> in kidney, bone and other tissues. Calcitonin increases <u>cAMP</u> in MCF 7 breast cancer cells. At 300-fold lower concentration calcitonin decreases <u>cAMP</u> in these cells. The decrease in <u>cAMP</u> is prevented by preexposure of cells to agents that interfere with inhibitory guanyl regulatory proteins. Intracellular <u>compartmentalization</u> of <u>cAMP</u> accumulation after calcitonin has been imaged after <u>microwave fixation</u> of cells. The <u>cAMP</u> accumulates initially along the plasma membrane but within 1 to 3 minutes accumulates much closer to the nucleus. </p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 43006-17 MD
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Study of Hyperparathyroidism: Etiology, Diagnosis and Treatment		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	G.D. Aurbach, M.D.	Chief MDB, NIDDK
OTHERS:	S.J. Marx, M.D.	Chief, Min. Metab. Sec. MDB, NIDDK
	K. Sakaguchi, M.D., PhD	Visiting Associate MDB, NIDDK
	A. Spiegel, M.D.	Chief MPB, NIDDK
	W. McKoy	Chemist MDB, NIDDK
COOPERATING UNITS (if any) Radiology Department, CC; (J. Doppman, T. Shawker) Surgery Branch, NCI; (DL Fraker, R. Alexandre) Department of Endocrinology, Univ. of Florence, Italy (ML Brandi)		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Endocrine Regulation Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER:
1.4	1.2	0.2
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrevduced type. Do not exceed the space provided.) <p> The project goal is the evaluation and treatment of <u>hyperparathyroidism</u>. Patients with persistent or recurrent hyperparathyroidism are referred for evaluation and treatment. Hereditary hyperparathyroidism in particular is under investigation in the hopes of delineating hereditary molecular abnormalities in glandular regulation, as exemplified in the <u>multiple endocrine neoplasia</u> syndromes. Evaluation ranges from epidemiologic studies of families to in-house clinical studies of patients and to in vitro analyses of excised tissue. Techniques currently being employed and improved include <u>radioimmunoassay</u> of parathyroid hormone, <u>ultrasonography</u>, (reoperative and intra operative) <u>radiothallium scanning</u>, <u>magnetic resonance imaging</u>, <u>CAT scanning</u>, <u>selective arteriography</u> and <u>selective venous sampling</u> for <u>parathyroid hormone</u>, parathyroid gland <u>cryopreservation</u> and autotransplantation, and transcatheter parathyroid gland infarction. In vitro evaluation of parathyroid and other endocrine tissue involves tissue culture, chemistry and determination of linkage with <u>DNA</u> or <u>RNA</u> probes. </p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 43008-11 MD
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) Vitamin D Resistance and Related Disorders		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sec. MDB, NIDDK
OTHERS:	J. Barsony, M.D.	Visiting Scientist MDB, NIDDK
	W. McKoy	Chemist MDB, NIDDK
COOPERATING UNITS (if any) Metabolism Unit, Beilinson Hospital, Betah Tiva, Israel (U.Liberman) Cell Biology Department, Baylor University (J.W. Pike) Biochemistry Department, University of Wisconsin, Madison (H.F. DeLuca) Hormone Action and Oncogenesis Section NCI (G. Hager)		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Mineral Metabolism Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.5	1.7	0.8
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided) <p>With recognition that vitamin D is the precursor for <u>1,25-dihydroxyvitamin D</u>, it has become possible to characterize defects in the activation (1-hydroxylation) of vitamin D and defects in the target action of activated (1,25-dihydroxy)vitamin D. We have demonstrated a broad spectrum of manifestations of <u>hereditary resistance</u> to 1,25(OH)2D ranging from infantile <u>rickets</u> with alopecia and no intestinal response to calciferols to adult onset <u>osteomalacia</u> with satisfactory intestinal response to high doses of calciferols and with no epidermal abnormalities. This syndrome usually results from a mutation in the gene for the vitamin D receptor. Skin fibroblasts from all subjects with hereditary resistance to 1,25(OH)2D display abnormalities in this effector system, and defects in many discrete steps of this pathway have been identified with these cells. Cells with mutations in the 1,25(OH)2D effector pathway can be used to explore mechanisms of calciferol action. They have been used to establish that the 1,25(OH)2D <u>receptor</u> mediates an extremely rapid (1-3 minutes) rise of <u>cyclic GMP</u> in response to 1,25(OH)2D3 and that certain receptor mutations compromise many receptor functions but allow another function to be retained normally. This establishes that 1,25(OH)2D receptors couple to different responses by distinct mechanisms.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 43009-07																								
PERIOD COVERED October 1, 1991 to September 30, 1992																										
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) Regulation of Mineral Metabolism																										
PRINCIPAL INVESTIGATOR (Last other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">S.J. Marx, M.D.</td> <td style="width: 35%;">Chief, Min. Metab. Sec.</td> <td style="width: 15%;">MDB, NIDDK</td> </tr> <tr> <td>OTHERS:</td> <td>W. McKoy</td> <td>Chemist</td> <td>MDB, NIDDK</td> </tr> <tr> <td></td> <td>G. Aurbach, M.D.</td> <td>Chief</td> <td>MDB, NIDDK</td> </tr> <tr> <td></td> <td>E. Friedman, M.D.</td> <td>Visiting Fellow</td> <td>MPB, NIDDK</td> </tr> <tr> <td></td> <td>A. Spiegel, M.D.</td> <td>Chief</td> <td>MPB, NIDDK</td> </tr> <tr> <td></td> <td>J. Norton, M.D.</td> <td>Head, Metabolism Sec.</td> <td>SB, NCI</td> </tr> </table>			PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sec.	MDB, NIDDK	OTHERS:	W. McKoy	Chemist	MDB, NIDDK		G. Aurbach, M.D.	Chief	MDB, NIDDK		E. Friedman, M.D.	Visiting Fellow	MPB, NIDDK		A. Spiegel, M.D.	Chief	MPB, NIDDK		J. Norton, M.D.	Head, Metabolism Sec.	SB, NCI
PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sec.	MDB, NIDDK																							
OTHERS:	W. McKoy	Chemist	MDB, NIDDK																							
	G. Aurbach, M.D.	Chief	MDB, NIDDK																							
	E. Friedman, M.D.	Visiting Fellow	MPB, NIDDK																							
	A. Spiegel, M.D.	Chief	MPB, NIDDK																							
	J. Norton, M.D.	Head, Metabolism Sec.	SB, NCI																							
COOPERATING UNITS (if any) EEB, SB, NIC, MPB Belvedere Medical Center - Carlisle, PA (J. Green), Dept of Physiol, Univ. of Manitoba, Canada (J.G. Friesen), Genetics Department - University (A. Bale) Department of Endocrinology - University of Florence Italy (ML Brandi)																										
LAB/BRANCH Metabolic Diseases Branch																										
SECTION Mineral Metabolism Section																										
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892																										
TOTAL MAN-YEARS 1.0	PROFESSIONAL 0.8	OTHER 0.2																								
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																										
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Disorders of mineral metabolism have been evaluated with methods extending from epidemiology to cellular and molecular biology. Two forms of <u>familial hyperparathyroidism</u> have been characterized in detail. <u>Familial hypocalciuric hypercalcemia</u> is an autosomal dominant trait associated with abnormal interactions with calcium in <u>parathyroid</u> and <u>kidney</u>. <u>Familial multiple endocrine neoplasia type 1 (FMEN1)</u> is an autosomal dominant trait causing hyperfunction of parathyroids, pancreatic islet and anterior pituitary. It is associated with gradual but abnormal proliferation of the tissues affected. <u>Genetic linkage studies</u> in a large kindred have localized the <u>FMEN1</u> gene to the long arm of chromosome 11. Plasma from affected persons shows high mitogenic activity upon cultured bovine parathyroid cells. This mitogenic activity in plasma may contribute to primary hyperparathyroidism in FMEN1. Analysis of blood and parathyroid tumor DNA has revealed that FMEN1 parathyroids often show clonal loss of alleles in the region of the FMEN1 gene on chromosome 11. Thus the FMEN1 gene functions as a <u>tumor suppressor gene</u>, analogous to the retinoblastoma gene. Analysis of sporadic <u>parathyroid adenomas</u> revealed that 25% showed allelic loss in a similar region. Thus the clonal inactivation of the FMEN1 gene may also be a contributing factor in many sporadic parathyroid adenomas. Tumors with allelic loss along chromosome 11 are being</p>																										

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 43200-13 MDB
PERIOD COVERED <u>October 1, 1991 through September 30, 1992</u>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Disorders of Immune Regulation in Systemic Lupus Erythematosus</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,		
P.I.: Others:	G.C. Tsokos J.E. Balow A. Patel	Guest Researcher Senior Investigator Biologist MDB, NIDDK MDB, NIDDK MDB, NIDDK
COOPERATING UNITS (If any)		
LAB/BRANCH <u>Metabolic Diseases Branch</u>		
SECTION <u>Kidney Disease Section</u>		
INSTITUTE AND LOCATION <u>NIDDK, NIH, Bethesda, MD 20982</u>		
TOTAL STAFF YEARS: <u>0</u>	PROFESSIONAL: <u>0</u>	OTHER: <u>0</u>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Project terminated.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH
SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 43201-08 MDB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Production and Characterization of Nephritic Factors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I.:	G.C. Tsokos	Guest Researcher	MDB, NIDDK
Others:	J.E. Balow	Senior Investigator	MDB, NIDDK
	R. Schwertz	Guest Researcher	MDB, NIDDK
	A. Patel	Biologist	MDB, NIDDK

COOPERATING UNITS (if any)

SUNY Medical Center, Syracuse, NY (Dr. R. Spitzer)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20982

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Project terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 43202-09 MDB
PERIOD COVERED October 1, 1991 through September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Regulation of Human Immune Response by Complement</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,		
P.I.: G.C. Tsokos Others: J.E. Balow	Guest Researcher MDB, NIDDK Senior Investigator MDB, NIDDK	
COOPERATING UNITS (if any)		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Kidney Disease Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20982		
TOTAL STAFF YEARS: 0	PROFESSIONAL: 0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <div style="height: 300px; border: 1px solid black; margin-top: 10px;"></div>		

Project terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43204-12 MDB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunosuppressive Drug Therapy in Lupus Glomerulonephritis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

P.I.:	J.E. Balow	Senior Investigator	MDB, NIDDK
Others:	H.A. Austin III	Medical Officer	MDB, NIDDK
	D.T. Boumpas	Visiting Scientist	MDB, NIDDK

COOPERATING UNITS (If any)

NIAMS (J. Klippel, P. Plotz, A. Steinberg, R. Wilder)
CC (E. Vaughan, C. Yarboro)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20982

TOTAL STAFF YEARS:

1.10

PROFESSIONAL:

1.10

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Pulse cyclophosphamide is more effective than prednisone alone in preventing renal failure in lupus nephritis. This study sought to define whether pulse methylprednisolone could equal pulse cyclophosphamide in preserving renal function, and whether there was a difference between long and short courses of pulse cyclophosphamide in preventing exacerbations of lupus.

Patients were treated with prednisone and randomized to receive concomitantly (a) pulse methylprednisolone monthly for 6 months, or (b) pulse cyclophosphamide monthly for 6 months, or (c) pulse cyclophosphamide monthly for 6 months followed by a maintenance regimen every 3 months for an additional two years. During the final 24 months of the study, all patients continue to receive low dose, alternate day prednisone.

Patients treated with pulse methylprednisolone had a higher probability of developing renal insufficiency than patients treated with the long course of pulse cyclophosphamide. In addition, patients treated with only a short course of pulse cyclophosphamide had a higher probability of major exacerbations of lupus than those treated with the extended course of pulse cyclophosphamide.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43205-15 MDB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Renal Biopsy Pathology in Systemic Lupus Erythematosus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

P.I.: J.E. Balow Senior Investigator MDB, NIDDK
Others: H.A. Austin III Medical Officer MDB, NIDDK

COOPERATING UNITS (if any)

Armed Forces Institute of Pathology, Washington, DC (T. Antonovych,
S. Sabnis); CC (E. Vaughan)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20982

TOTAL STAFF YEARS:

0.15

PROFESSIONAL:

0.15

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Diverse pathogenetic factors are operant in systemic lupus erythematosus and lead to different forms of lupus nephritis. Detailed analysis of renal biopsy pathology is being conducted on specimens from patients with systemic lupus erythematosus.

Biopsies are classified by standard major category of lupus nephritis, as well as scored on a semi-quantitative scale for specific histologic changes which indicates the extent and severity of active inflammatory lesions and chronic atrophic, fibrosing and sclerosing features. The patterns of immune complex deposition and lymphoid cell interaction with different segments of the nephron are being investigated by immunohistologic techniques and electron microscopy.

These approaches have facilitated the analysis of the effects of various types of immunosuppressive agents used to halt the progression of lupus nephritis and they will enhance our understanding of the pathogenesis of lupus nephritis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43211-08 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Morphology of Renal Lesions in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: L. Striker, M.D., Chief RCBS

Others: G. Striker, M.D., Senior Investigator

C. Pesce, M.D., Visiting Associate

K. Schmidt, M.D., Interinstitute Genetics Fellow

COOPERATING UNITS (if any)

Epidemiology and Clinical Research Branch, NIDDK, Phoenix Arizona
(Peter Bennett)

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

0.90

PROFESSIONAL:

0.90

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) Human X (b) Human (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The weighted mean of the area and a size-class distribution of the glomeruli were calculated with a computer-assisted planimeter in a series of autopsy kidney specimens of diabetic and non-diabetic Pima Indians. These morphometric variables were also related to the severity of histologic glomerular lesions, graded on a four class scale. Glomerular size did not differ in the two groups, nor were differences in the size distribution discernible among the classes of histologic lesions. A similar pattern was found in a Caucasian population with NIDDM. These data are in contrast to IDDM where there is an early increase in glomerular size, followed by a late decrease in glomerular size as end-stage renal disease supervenes. The glomeruli of diabetic Pima Indians did not decrease in size at the stage of diffuse sclerosis, a finding that contrasts with what is commonly seen in other types of kidney disease, especially those associated with vascular lesions. Comparisons between the size of the glomeruli in autopsy specimens from caucasian and black individuals devoid of renal disease was also undertaken.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43214-08 MD

PERIOD COVERED

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Glomerular Cells Derived From Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I.: L. Striker, M.D.
Others: G. Striker, M.D., Senior Investigator
S. Elliot, Ph.D., GS-12
E. Peten, M.D., Guest Worker
M. Carome, M.D., Guest Worker

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

0.22

PROFESSIONAL:

0.22

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Current models of glomerulosclerosis (GS) have yielded little information about the cellular and molecular abnormalities that are critical in the initiation and progression of this disease. The complexity of the kidney and glomerulus make isolation and examination of pure cultured populations of glomerular cells an attractive method for beginning to answer these questions. Unfortunately other models of GS involve extrarenal causes of glomerular injury, including hormonal or cellular events. Because of this, it is quite likely that glomerular cells isolated from these models will not maintain the abnormal behavior in vitro which led to the development of GS in vivo.
Mice transgenic for bovine growth hormone develop progressive glomerulosclerosis. We have isolated lines of glomerular mesangial from transgenic mice and have isolated lines of mesangial, endothelial cells and epithelial cells from their normal littermates. Our data from the in vivo model indicates that proliferation of mesangial glomerular cells is an early event in the development of GS in transgenic mice, we will evaluate several growth factors on the behavior of individual cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRANURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43221-06 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology of Insulin Receptors in Glomerular Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I.: L. Striker, M.D., Chief RCBS

Others: G. Striker, M.D., Senior Investigator

S. Elliot, Ph.D., GS-12

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Terminated

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43222-07 MDB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis of Murine Lupus Nephritis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

P. I.:	H. A. Austin	Medical Officer	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	D. T. Boumpas	Visiting Scientist	MDB, NIDDK
	A. D. Patel	Biologist	MDB, NIDDK

COOPERATING UNITS (If any)

Armed Forces Institute of Pathology; Washington, DC (T. Antonovych and S. Sabnis).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.25

PROFESSIONAL:

0.25

OTHER:

1.00

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Investigations of the pathogenesis and treatment of lupus nephritis are facilitated by the availability of inbred strains of mice that develop disease similar to human systemic lupus erythematosus. The natural evolution of the diverse histologic features of murine lupus nephritis has been studied to delineate the types of glomerular and tubulointerstitial lesions.

Innovative treatment strategies will be studied to refine our approach to this disease. The impact of biologic response modifiers on immunologic features will be investigated as well. Clinical, histologic and immunologic outcome parameters will be evaluated including detailed studies of renal morphology, and the characteristics of spleen lymphocytes employing flow cytometry, measures of immunoglobulin gene expression, and in vitro assays of alterations in humoral and cell mediated immune regulation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43224-06 MDB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Membranous Lupus Nephropathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

PI:	H. A. Austin	Medical Officer	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	K. MacKay	Expert	MDB, NIDDK

COOPERATING UNITS (if any)

CC (E. Vaughan); NIAMS (J. Klippel); Stanford University; Stanford, CA (B. Myers). Armed Forces Institute of Pathology, Washington, DC (T. Antonovych and S. Sabnis).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.50

PROFESSIONAL:

0.50

OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It is currently unknown whether therapeutic intervention will alter the course of membranous lupus nephropathy. In the present study, the efficacy and toxicity of three immunosuppressive drug regimens administered over a 12 month period will be evaluated in patients with membranous lupus nephropathy. Detailed tests of renal function (including radiolabelled compounds for glomerular filtration and renal plasma flow rates), glomerular permselectivity (using fractional clearance of graded dextrans) and kidney biopsy morphology will be performed at the beginning and end of treatment.

Patients with systemic lupus erythematosus, nephrotic range proteinuria and biopsy documented membranous nephropathy will be randomized to receive: a) alternate day prednisone alone (control group), b) alternate day prednisone plus intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses, or c) alternate day prednisone plus oral cyclosporin A up to 200 mg per square meter body surface area daily. Lupus disease activity, renal function tests and drug toxicities will be monitored closely. Analysis will include comparison of the numbers of favorable outcomes of glomerular filtration rate, renal plasma flow, permselectivity, glomerular pathology and drug-related toxicities appearing in each treatment group.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43225-05 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glomerular Lesions in Mice Transgenic for Growth Hormone

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I.: L. Striker, M.D.
Others: G. Striker, M.D., Senior Investigator
S. Elliot, Ph.D., GS-12
C. Yang, M.D., Visiting Associate
E. Peten, M.D., Guest Worker

COOPERATING UNITS (if any)

John Kopchick, Ph.D., University of Ohio

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

1.10

PROFESSIONAL:

1.10

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Increased glomerular size occurs in the presence of normal maturation, following unilateral nephrectomy in humans and animals, and in disease states such as diabetes mellitus. There are abnormalities in the circulating levels of GH in some diseases associated with increases in glomerular extracellular matrix and cell number, such as diabetes mellitus. The availability of transgenic mouse strains expressing elevated levels of GH provides an opportunity to study the renal effects of chronic hormone exposure. Progressive glomerulosclerosis leading to kidney failure develops in the GH transgenic mice. Mice transgenic for GH molecules containing mutations have also been examined in order to elucidate the domains of GH that may specifically code for genes responsible for glomerulosclerosis. We found an upregulation of mRNA for extracellular matrix components in the kidneys of the GH animals. This upregulation persisted late in the course of the disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
201 DK
43227-05 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of IGF-1 in the Biology of Mouse Glomerular Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I. L. Striker, M.D., Chief RCBS
Others: G. Striker, M.D., Senior Investigator
S. Elliot, Ph.D., GS-12
E. Peten, M.D., Guest Worker

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

0.70

PROFESSIONAL:

0.70

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The IGF-I axis has been implicated in the development of renal complications of diabetes. Glomerulosclerosis of diabetes is characterized by mesangial cell proliferation and accumulation of extracellular matrix in the mesangium. This suggests that there may be an intrinsic defect of mesangial cell behavior. Non-obese diabetic mice (NOD) develop glomerular lesions early after the onset of IDDM. We developed new lines of mesangial cells derived from these animals to study the role of the IGF-I axis in a model of spontaneous diabetes. We examined the IGF-I receptor, IGF-I production, and IGF-I binding proteins of the NOD mice and compared them to control mesangial cells. We previously demonstrated the presence of IGF-I receptors and the synthesis of IGF-I in glomerular mesangial cells. In the current study we examined mouse glomerular endothelial and epithelial cells in culture for IGF-I receptors. [¹²⁵I]IGF-I specifically bound to the cell surface of both cell types. Maximum specific binding, 0.141 B/F for endothelial cells and 0.301 B/F for epithelial cells, was obtained at 22 C after 150 min incubation. The estimated Kd values were 2.25x10 for endothelial cells and 1.5x10 for epithelial cells. Cross-linking studies showed a single band of radioactivity with an estimated mol. wt. of 145kD, consistent with the α-subunit of the IGF-I receptor. Radiolabelled IGF-I was not degraded by either cell types. These findings suggested a paracrine role of IGF-I in the glomerulus.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43228-05 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology of Human Mesangial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I.: L. Striker, M.D., Chief RCBS

Others: G. Striker, M.D., Senior Investigator

S. Elliot, Ph.D., GS-12

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human X (b) Human (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Inactive

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43231-04 MDB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Idiopathic Membranous Nephropathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

P.I.:	H. A. Austin	Medical Officer	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	K. MacKay	Expert	MDB, NIDDK

COOPERATING UNITS (If any)

Stanford University, Stanford, CA (B. Myers); CC (E. Vaughan); Armed Forces Institute of Pathology; Washington, DC (T. Antonovych and S. Sabnis).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.50

PROFESSIONAL:

0.50

OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Patients with idiopathic membranous nephropathy are being studied to evaluate the efficacy and toxicities of the addition of intermittent cyclophosphamide or daily oral cyclosporin A to alternate day oral corticosteroid therapy. Efficacy will be judged by determinations of effective renal plasma flow, glomerular filtration rate and glomerular capillary wall permselectivity performed with dextran and urine protein (albumin and immunoglobulin) clearance techniques. Kidney biopsy morphology (including morphometric analysis) will be examined at the beginning and at the end of treatment as part of detailed studies of structure-function relationships and the efficacy of various therapeutic modalities.

Patients with membranous nephropathy and 2 or more grams per day of proteinuria will be treated with alternate day prednisone and will be randomized to receive: a) no additional therapy (control group), b) intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses, or c) oral cyclosporin A up to 200 mg per square meter body surface area daily for a total of 11 months. Analysis will include comparison of the number of favorable outcomes of glomerular function and pathology as well as drug-related toxicities observed in each treatment group at the end of the 12 months of study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 43232-03 MD
PERIOD COVERED 10/01/91 thru 9/30/92		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Renal Lesions in the Ablation Model</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, P.I.: L. Striker, M.D. Others: G. Striker, M.D., Senior Investigator C. Pesce, M.D., Visiting Associate		
COOPERATING UNITS (if any) Washington University, Saulo Klahr, M.D.		
LAB/BRANCH Metabolic Diseases		
SECTION Renal Cell Biology, Bld 10 Rm.3N-110		
INSTITUTE AND LOCATION NIDDK, NIH Bethesda, MD 29892		
TOTAL STAFF YEARS: 0.55	PROFESSIONAL: 0.55	OTHER: 0
CHECK APPROPRIATE BOX(ES) (a) Human (b) Human X (c) Neither (al) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Subtotal nephrectomy in rats, the 5/6ths ablation model, leads to progressive loss of kidney function and chronic renal failure. We postulated that the progressive glomerular lesions were due to an early increase in the turnover of glomerular resident cells. This leads to abnormal glomerular growth, with an increase in the glomerular volume which was detectable using morphometric measurements. We have performed autoradiographic studies, using ³ H-thymidine, and found that within two days following subtotal nephrectomy there was an increase in the glomerular cell mitotic index, as well as an increase in the turnover of the cells in the arterial wall. These findings suggest that dysregulation of cell growth is an early event in the development of glomerulosclerosis following reduction in renal mass.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
201 DK
43233-03 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of IGF-1 Binding Proteins in Mesangial Cells in vitro

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I.: L. Striker, M.D.

Others: G. Striker, M.D., Senior Investigator

S. Elliot, Ph.D.

COOPERATING UNITS (if any)

none

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human X (b) Human (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Terminated

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 43234-03 MDB
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PERIOD COVERED
October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Interactions Between Transforming Growth Factor (TGF- β) and Glomeruli

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

P.I.: **K. MacKay Expert** **MDB, NIDDK**
Others: **A. R. Robbins** **LBM, NIDDK**

COOPERATING UNITS (if any)
D. Danielpour (Laboratory of Chemoprevention, NCI)

LAB/BRANCH
Metabolic Diseases Branch

SECTION
Kidney Disease Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS: 0.80	PROFESSIONAL: 0.80	OTHER:
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CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Increased glomerular cellularity and accumulation of extracellular matrix material are prominent histologic findings in a number of clinical and experimental glomerular diseases. Transforming growth factor- β (TGF- β) has been identified as a potentially important modulator of glomerular pathology based in its demonstrated ability to regulate proliferation and extracellular matrix synthesis by cultured glomerular cells. In addition, we have previously demonstrated that normal rat glomeruli contain high levels of TGF- β 1 and TGF- β 2 and that glomeruli possess unique TGF- β binding proteins or receptors.

The goal of these studies is to better understand the actions and mechanisms of action of TGF- β in the glomerulus. The current focus of these studies is on the TGF- β binding proteins and/or receptors which are present in glomeruli.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43235-03 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glomerular Lesions in Non-Obese Diabetic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title.

P.I. L. Striker, M.D., Chief RCBS
Other: G. Striker, M.D., Senior Investigator
T. Doi, M.D., Visiting Scientist
D. Yang, M.D., Visiting Associate

COOPERATING UNITS (if any)

M. Hattori, M.D., Joslin Diabetes Center, Boston, MA

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

0.75

PROFESSIONAL:

0.75

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

NOD mice spontaneously develop insulin-dependent diabetes mellitus (IDDM) secondary to immunologically-mediated beta cell destruction in pancreatic islets. Shortly after the appearance of diabetes, NOD mice developed renal lesions consisting of diffuse mesangial sclerosis, thickening of glomerular basement membranes, and albuminuria. These lesions closely mimic those of IDDM in humans. Morphometric analysis showed that the kidney weight and glomerular size were increased in diabetic mice, compared to non-diabetic mice, and that the ratio glomerular volume/kidney weight was elevated in diabetic mice. These findings suggest that this disproportionate increase in glomerular size may play an important role in the development of diabetic nephropathy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43236-02 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Advanced Glycosylation End Products, Effects on Mesangial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I.: L. Striker, M.D.

Others: G. Striker, M.D., Senior Investigator

T. Doi, M.D., Visiting Scientist

COOPERATING UNITS (if any)

H. Vlassara, M.D., Picower Institute, Manhasset, NY

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

0.60

PROFESSIONAL:

0.60

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

End-stage glomerulosclerosis constitutes a major complication of diabetes mellitus. The fact that the glomerular lesions of both type I and type II diabetes are similar suggests that abnormalities in glucose metabolism may participate in their development. Hyperglycemia leads to the accumulation of advanced glycosylation end-products. These products participate in abnormal, non-metabolizable cross-linking of extra-cellular matrix components. Their accumulation may contribute to the sclerosis observed in diabetics. AGEs trigger a large number of biological reactions which are mediated by surface receptors that have been characterized on macrophages, endothelial cells, and human and rat mesangial cells.

Mesangial cells plated on various glycosylated extracellular matrix components produce an increased amount of fibronectin. Using normal mouse mesangial cells, we investigated the effect of AGE on the synthesis of the basement membrane components. Cells plated on AGE showed increased levels of the following mRNAs using the RNase protection assay: collagen type IV, proteoglycan heparan sulfate, and laminin A and B chains. We also found an increased release of collagen type IV into the medium. The rate of transcription, measured by nuclear run-off assays, was also stimulated in cells plated on glycosylated bovine serum albumin. AGE receptor antibodies inhibited the observed increase in mRNAs. Antibodies to PDGF abrogated the AGE response. These observations provide further evidence that the accumulation of extracellular matrix components in diabetics is regulated at the gene level, and is correlated with an increase in mesangial cell turnover.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43237-02 MD

PERIOD COVERED

10/1/91 to 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Production of Metalloproteinases and TIMP-1 by Glomerular Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P. I.: L. Striker, M.D.

Others: G. Striker, M.D., Senior Investigator
Michael Carome, M.D., Guest Researcher
Emmanuel Peten, M.D., Guest Researcher

COOPERATING UNITS (if any)

Laboratory of Pathology, NCI, Dr. Wm. Stetler-Stevenson
Walter Reed Army Medical Center, Dr. J. Moore

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

0.80

PROFESSIONAL:

0.80

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Glomerulosclerosis occurs in a large number of human kidney diseases, including diabetic nephropathy. It consists of the accumulation of extracellular matrix (ECM) within the glomerulus. Sclerosis may be mediated by dysregulation of both synthesis and degradation of ECM. A large family of matrix metalloproteinases as well as tissue inhibitors of metalloproteinase (TIMPs) play a role in the degradative process. Our preliminary work reveals that: 1) normal mouse mesangial cells in culture secrete a 72 kD and a 92 kD gelatinase (type IV collagenase) as well as TIMP1; 2) mesangial cells derived from NOD mice and mice transgenic for bovine growth hormone (bGH) secrete only the 72 kD gelatinase; and 3) TIMP1 mRNA levels are modulated by cell density.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43238-02 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gene Expression in Microdissected Mouse Glomeruli

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I.: L. Striker, M.D.
Others: G. Striker, M.D., Senior Investigator
E. Peten, M.D., Guest Researcher
Michael Carome, M.D., Guest Researcher

COOPERATING UNITS (if any)

A. Garcia-Perez, Ph.D., NHLBI

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:
0.70

PROFESSIONAL:
0.70

OTHER:
0

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this study is to determine the phenotype of extracellular matrix molecules in normal adult mouse glomeruli. We used the increased sensitivity afforded by the polymerase-chain reaction to assess type I and type IV collagen mRNA in freshly microdissected normal adult mouse glomeruli. RT-PCR reactions for mRNA encoding these components were also performed using mesangial cell lines previously isolated from the same strain of mice. Type IV collagen mRNA was easily detectable in normal adult mouse glomeruli as well as in the cell lines. On the other hand, type I collagen mRNA was not detected in the glomeruli, despite increasing the number of PCR cycles from 25-45 (roughly a 1000 fold increase in sensitivity). Assays using competitive PCR were also developed. Utilizing the same primers, type I collagen mRNA was easily demonstrable in two lines of mouse mesangial cells. These experiments support data in both humans and experimental models which failed to demonstrate type I collagen by immuno-fluorescence microscopy in normal glomeruli, whereas type IV collagen was present in large amounts. The current study provides evidence that the expression of types I and IV collagen in normal glomeruli is regulated at the pretranslational level in vivo.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43239-02 MDB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of Glucocorticoids on T Cell Activation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

P. I.:	D. T. Boumpas	Visiting Scientist	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	S. S. Ahuja	Visiting Associate	MDB, NIDDK
	F. Paliogianni	Visiting Fellow	MDB, NIDDK
	J. P. Balow	Summer Student	MDB, NIDDK

COOPERATING UNITS (If any)

Laboratory of Biochemistry, NCI, NIH (C. B. Klee)
Section on Immunology, NIAAA, NIH (R. L. Kincaid)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.10

PROFESSIONAL:

2.00

OTHER:

0.10

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Activation of T cells is a complex process involving cell membrane, cytoplasmic and nuclear events. These events enable T cells to proliferate and exert their immunoregulatory function. The goal of these studies is to better understand the calcium-dependent pathway of T cell activation and define the effects of biologic agents used for the therapy of immune mediated renal diseases (glucocorticoids, cyclosporine A) and those mediators produced at the site of inflammation (prostaglandin E2, transforming growth factor-beta or TGF- β) on T cell activation.

Findings to date indicate that glucocorticoids inhibit IL-2 gene transcription by interfering with the binding of nuclear factors AP-1 and NF-AT on the human IL-2 promoter. Glucocorticoids inhibit both T cell antigen receptor and IL-2 receptor mediated proliferative signals (tyrosine phosphorylation, production of transcription factors, expression of lymphokine genes, Rb protein phosphorylation). Prostaglandin E2 also downregulates T cell activation and proliferation by inhibiting tyrosine phosphorylation and nuclear transcription of IL-2, while TGF- β inhibits only IL-2 receptor mediated proliferative signals.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43240-02 MDB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transcriptional Regulation of Immunoglobulin Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

P. I.:	D.T. Boumpas	Visiting Scientist	MDB, NIDDK
Others:	F. Paliogianni	Visiting Fellow	MDB, NIDDK
	J. P. Balow	Summer Student	MDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.80

PROFESSIONAL:

.70

OTHER:

0.10

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Reduction of pathogenic autoantibodies is the main objective in the therapy of immune mediated renal diseases. Although rates of immunoglobulin synthesis can be regulated at multiple levels, most attention has been focused on transcription, as this seems to be the limiting step in most situations that have been examined. The goal of these studies is to examine the ability of a variety of agents (known to regulate the activation, proliferation and differentiation of B cells) to modulate the nuclear transcription of immunoglobulin heavy and light chain genes. These agents include antibodies to surface immunoglobulin, cytokines (interleukins, interferon, transforming growth factor), bacterial mitogens, pharmacologic agents (ionomycin, phorbol esters, prostaglandins) and immunosuppressive drugs (glucocorticoids, cyclosporin, cyclophosphamide). The mechanism of action of these agents will be explored by examining their effects on immunoglobulin gene transcription. Particular emphasis will be placed in detecting similarities, differences, synergism or antagonism in their effects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43241-01 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Pathogenesis of Glomerulosclerosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I.: L. Striker, M.D.

Others: G. Striker, M.D., Senior Investigator

S. Elliot, Ph.D., GS-12

C. Yang, M.D., Visiting Associate

E. Peten, M.D., Guest Worker

M. Carome, M.D., Guest Worker

COOPERATING UNITS (if any)

Walter Reed Army Medical Center, Dr. J. Moore

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human ☒ (b) Human ☒ (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The renal cell biology section is interested in the cellular and molecular mechanisms leading to glomerular scarring, the emphasis being on non-immune diseases. The kidney disease of diabetes mellitus is the major disease studied. Our hypothesis is that glomerulosclerosis results from abnormalities in the rate of proliferation and matrix turnover by resident glomerular cells. This is being investigated in vitro, using clonal lines of glomerular cells, and in vivo using transgenic mice. We have developed a new technique to quantitatively study the synthesis and degradation of glomerular matrix components in vivo. The method consists of microdissection of single mouse and human glomeruli, reverse transcription in situ. This was followed by the polymerase-chain reaction (PCR). We have developed a method to quantitate PCR, so that we can examine the relative amounts of the different mRNAs coding for the various basement membrane collagens and the enzymes that degrade collagens. This technique is now being applied to extracellular matrix turnover in glomeruli of mice transgenic for growth hormone and to human glomeruli obtained from nephrectomy specimens.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK
43242-01 MD

PERIOD COVERED

10/01/91 thru 9/30/92

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Degradation of Extracellular Matrix in Human Glomeruli

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

P.I.: L. Striker, M.D.

Others: G. Striker, M.D., Senior Investigator
Michael Carome, M.D., Guest Researcher

COOPERATING UNITS (if any)

Laboratory of Pathology, NCI, Dr. Wm. Stetler-Stevenson
Walter Reed Army Medical Center, Dr. J. Moore

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bld 10 Rm.3N-110

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, MD 29892

TOTAL STAFF YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human X (b) Human (c) Neither
(a1) Minors
(a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Degradation of collagens and non-collagenous components of the glomerular extracellular matrix is believed to play an important role in the turnover of normal glomerular basement membranes, and in the pathogenesis of glomerulosclerosis. A new method combining microdissection of human glomeruli and competitive PCR has been utilized to examine glomeruli isolated from nephrectomy specimens. This method allows the detection of cDNA molecules in individual glomeruli, because of a detection range in the range of 10^{-4} attomoles. Preliminary results from glomeruli isolated from human nephrectomies indicate that the method is sensitive, reproducible, and allows comparison between patients.

ANNUAL REPORT OF THE LABORATORY OF THE DIABETES BRANCH

National Institute of Diabetes and Digestive and Kidney Diseases

Investigators in the Diabetes Branch conduct clinical investigation and basic scientific research with special emphasis on understanding the mechanism of action of insulin and the causes of diabetes mellitus. Towards this end, a multi-disciplinary approach has been applied, involving techniques of molecular biology, cell biology, biochemistry, and clinical physiology. Other projects include studies of hormones and other messenger molecules important to the regulation of growth and development, especially growth hormone and insulin-like growth factors I and II. In addition, there is an active research program with respect to acromegaly, a clinical disease caused by the overproduction of growth hormone.

Insulin receptors

The insulin receptor is a heterotetrameric glycoprotein located on the cell surface. It is encoded by a single gene that encodes a precursor molecule. The precursor undergoes multiple post-translational processing steps including proteolytic cleavage to yield two different types of subunits: an α -subunit that contains the insulin binding site and a β -subunit that is a tyrosine specific protein kinase. When insulin binds to the extracellular domain of the receptor, this activates the tyrosine kinase associated with the intracellular domain of the receptor. Activation of the tyrosine kinase plays a necessary role in mediating insulin action. Several projects in the Diabetes Branch pertain to various aspects of insulin receptor biosynthesis and insulin receptor function.

To study the regulation of the expression of the insulin receptor gene, the DNA in the 5' flanking region of the human and mouse insulin receptor genes have been cloned. Deletion analysis has been employed to identify a 70-base pair region of the gene that contains the majority of the promoter activity. In addition, a weak enhancer has been identified upstream of the promoter. Further investigations are directed towards identifying other regulatory domains that may play a role in the developmental and endocrine regulation of the expression of the insulin receptor gene. For example, there are three consensus binding sites for the transcription factor C/EBP. Expression vectors containing these sequences can be transactivated by an expression vector for the transcription factor C/EBP. A sequence in the first intron has been identified that is required for the 10-fold induction of the insulin receptor gene during adipocyte differentiation.

In another project, several steps involved in post-translational processing of the insulin receptor during biosynthesis are being investigated. In particular, N-linked glycosylation, O-linked glycosylation, and fatty acylation are being studied. The role of N-linked glycosylation is being investigated by site-directed mutagenesis to eliminate the N-linked glycosylation sites. By expression of the cDNAs encoding these mutant receptors, it may be possible to determine the functional role of the N-linked glycosylation. Progress has also been made toward identifying the O-linked glycosylation site in the β subunit.

The role of the tyrosine kinase in mediating insulin action is also being investigated. A 120 kilodalton glycoprotein in rat liver membranes has been identified to serve as a substrate for phosphorylation by the insulin receptor. This glycoprotein has been immunoaffinity purified, a partial amino acid sequence has been determined, and a cDNA encoding this glycoprotein has been obtained. This protein substrate, pp120, appears to be identical to a previously identified enzyme that has the property of hydrolyzing ATP and GTP. The effect of phosphorylation to regulate the enzymatic activity of this protein is presently being investigated. By expressing the cDNA through transfection, it should be possible to determine the physiological role of this protein, and what role the protein may have in mediating the effect of insulin upon target cells.

In addition, the gene encoding rat pp120/ecto-ATPase has been cloned. It contains nine exons and spans approximately 15,000 b.p. of DNA. Exon 7 undergoes variable splicing yielding two isoforms of mRNA. The isoform lacking exon 7 encodes a short form of the protein in which the cytosolic domain is truncated to only 10 amino acids. This short isoform lacks the phosphorylation sites that are present in the long isoform of the protein.

In addition, as part of a long term collaboration with scientists at the University of Geneva, studies are underway to explore the mechanism and significance of receptor-mediated endocytosis, receptor internalization, and recycling. It has been shown that tyrosine phosphorylation is required for redistribution from the microvilli to clathrin coated pits, and as a result for ligand-stimulated endocytosis.

Considerable progress has been made in identifying mutations in the insulin receptor gene, and elucidating the role of this type of genetic defect in causing human disease. Multiple different mutations have been identified in the insulin receptor gene, these fall into five classes: class 1, mutations that inhibit insulin receptor biosynthesis, frequently by decreasing levels of insulin receptor mRNA; class 2, mutations that impair the transport of mutant receptors to the cell surface; class 3, mutations that decrease the affinity with

which insulin is bound to the receptor; class 4, mutations that impair tyrosine kinase activity; class 5, mutations that accelerate receptor degradation, apparently by inhibiting recycling of internalized receptors back to the plasma membrane. Most of these mutations have been identified in patients with relatively rare genetic syndromes associated with severe insulin resistance and acanthosis nigricans. At present, studies are underway to determine the prevalence of this type of mutation, and also to investigate the possibility that this type of mutation may contribute to the pathogenesis of more common diseases such as noninsulin-dependent diabetes mellitus and polycystic ovary disease. In addition, studies have been initiated to treat these insulin resistant patients with IGF-I to determine whether it has therapeutic advantages as compared to insulin.

One allele of the insulin receptor gene in cultured 3T3-L1 cells has been inactivated by the technique of homologous recombination. Inactivation of the insulin receptor gene was associated with a marked impairment in the ability of the 3T3-L1 cells to differentiate into adipocytes in vitro. This suggests an important role for the insulin receptor in regulating the development and differentiation of fat cells. The method of homologous recombination is now being applied in an effort to inactivate the insulin receptor gene in embryonic stem cells. Eventually, this approach should enable the production of transgenic mice with mutations in the insulin receptor gene and also other genes relevant for glucose homeostasis and diabetes. The production of animal models of disease by this technique should be very useful in studying both the causes and treatment of diabetes.

Insulin-like growth factors

Mammalian IGF-I genes are composed of at least 6 exons. The mature peptide is encoded by exons 3 and 4. As a result of variable splicing and the use of alter polyadenylation sites, there are multiple species of IGF-I mRNA ranging in size from 0.8 - 7.5 kb in length. Transcripts containing exon 2 are more sensitive to regulation by growth hormone. Transcripts containing exon 1 are expressed ubiquitously in all tissues, but are less sensitive to regulation by growth hormone.

Unilateral nephrectomy results in compensatory enlargement of the remaining kidney. In adult rats this primarily induces hypertrophy of existing cells and is regulated by GH, since isolated GH deficiency prevents the hypertrophy and no increase in IGF-I gene expression is seen, suggesting that the GH effect may either be direct or via elevated systemic IGF-I levels. In contrast, compensatory hypertrophy in immature animals primarily involves hyperplasia and is associated with increased kidney IGF-I gene expression. This increased expression is not GH-dependent and is probably causative in the hyperplastic process. The effects of IGF-I on renal

hemodynamics can be suppressed by simultaneous infusion of a kinin-receptor antagonist. It is possible that IGF-I plays a role in the pathogenesis of diabetic kidney disease.

Thus, the IGFs play an important role in normal development and certain pathological states and future studies will hopefully elucidate the molecular mechanisms underlying these diverse processes involving IGF-I action.

Insulin-like growth factor receptors

The biological effects of IGF-I are mediated through a cell surface receptor, a heterotetrameric glycoprotein that is homologous to the insulin receptor. A cDNA encoding a portion of the IGF-I receptor has been cloned from rat granulosa cells. In addition, partial length cDNAs encoding receptors in this family have been cloned from *Xenopus* embryos. A portion of the 5' flanking region of the IGF-I receptor gene has been cloned from the rat. The promoter region of the gene has been identified. This region contains a unique transcription initiator sequence with a single start site, previously found in a number of developmentally regulated genes. The promoter region lacks a TATA box, but contains putative binding sites for several transcription factors: Sp1, ETF, GCF, AP2, and Wilm's tumor binding sites.

The expression of the IGF-I receptor gene is regulated under many physiological and pathophysiological conditions. The developmental regulation of IGF-I receptor gene expression demonstrated tissue-specific changes; in most tissues there was a dramatic postnatal decrease. Brain demonstrated the highest prenatal levels of receptor gene expression.

Insulinopenic diabetes is associated with early changes in renal function. These include enlargement of the kidneys and elevated levels of glomerular filtration rate and renal plasma flow. Both GH and IGF-I can cause these changes, yet insulinopenic diabetes is often associated with reduced serum GH and IGF-I levels in the rat. In contrast, the binding of IGF-I and IGF-II to kidney membranes was increased, under these conditions. Using antisense RNA probes for these receptors, we have shown that expression of the receptor gene is indeed elevated within 24 hours of induction of insulinopenic (streptozotocin-induced) diabetes. This elevated receptor level may also contribute to the early renal changes in diabetes.

Compensatory renal enlargement rapidly follows unilateral nephrectomy. In immature rats, the enlargement of the contralateral kidney is primarily hyperplastic and is associated with increased steady-state levels of IGF-I receptor mRNA as well as increased IGF-I binding to kidney membranes. In contrast, in adult rats, the enlargement is associated with hypertrophy and constant IGF-I receptor mRNA levels, suggesting

that the IGF-I receptor mediates hyperplasia in immature rats but is not involved in the hypertrophy characteristics of adult animals.

In addition, studies have been initiated to define structure-function relationships of the IGF-I receptor. Toward that end, the key lysine in the ATP-binding site was mutated to an alanine. This mutation inhibited receptor tyrosine kinase activity, and also inhibited the ability of the receptor to mediate the effects of IGF-I to stimulate DNA synthesis, 2-deoxyglucose uptake, and phosphatidyl inositol-3-kinase activity. These observations suggest that receptor tyrosine kinase activity is required for the receptor to mediate biological activity.

Insulin-receptor related receptor

A gene has been identified that is homologous to the genes encoding the receptors for insulin and IGF-I. Studies have been initiated to investigate this third receptor, the so-called insulin-receptor related receptor (IRR). We have cloned the cDNA encoding mouse IRR, and also the murine gene. The IRR will be expressed by transfection of the cDNA. The recombinant IRR will be used to identify the endogenous ligand for the receptor. In addition, efforts are under way to inactivate murine IRR by homologous recombination. These studies may allow for elucidation of the physiological role of the IRR.

Growth hormone and acromegaly

A long term follow-up study of patients with acromegaly has been conducted in the Diabetes Branch over the past 25 years. Several modes of therapy have been employed, including radiation therapy, trans-sphenoidal hypophysectomy, and more recently treatment with drugs such as bromocriptine and somatostatin analogs. Recent studies have demonstrated that somatostatin analogs can be extremely useful in lowering growth hormone levels in patients with acromegaly. In addition, this agent can be useful in reducing hormonal hypersecretion caused by tumors of the pituitary that secrete thyroid stimulating hormones (TSH) and also in glucagon secreting tumors of the pancreas. Unfortunately, somatostatin analogs have a side effect of causing the patients to develop thickened bile. Because of the previous observation that patients with somatostatin secreting tumors have a high prevalence of gallstones, it seemed important to determine how frequently treatment with somatostatin analogs would also cause this complication.

Of patients who have received long-term treatment with the long-acting somatostatin analog, 70% of the patients with acromegaly have developed gallbladder sludge and 20% have

deveveloped gallstones. Patients currently enrolled in this study who have persistent sludge or gallstones will be treated with ursodeoxycholate, a drug with the potential to prevent or to reverse the formation of sludge and gallstones.

Quantitation of Insulin Action *in vivo*

Euglycemic insulin clamps combined with either positron emission tomography (PET) or magnetic resonance imaging (MRI) are being used to measure insulin action *in vivo*. This approach allows for study of regional effects of insulin and IGF-I upon metabolism of specific tissues and organs. These approaches are being used to characterize pathophysiological mechanisms in hypoglycemic and diabetic states.

In addition, a less invasive technique is being evaluated: minimal model analysis of the frequently sampled intravenous glucose tolerance test. Preliminary data suggest that minimal model analysis underestimates the importance of insulin in regulating glucose homeostasis. Efforts will be made to improve the mathematical analysis in order to obtain improved measures of insulin action *in vivo*.

Regulation of gene expression

One of the central questions in developmental biology relates to how genes are regulated in a tissue-specific manner. For example, the insulin gene is expressed at high level in the pancreatic beta cell while the gene is essentially shut off in other tissues. In order to define the molecular mechanisms involved in this type of regulation, the beta globin locus has been used as a model system. Thus far, the chicken beta globin locus has been analyzed using both transient expression in cultured cells and also expression in transgenic mice. These studies have identified multiple regulatory elements including a locus control region that allows for positron-independent copy number-dependent expression of the gene.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 47001-10 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (20 characters or less. Title must fit on one line between the borders)

Phosphorylation of Insulin and IGF-I Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. (Name, title, laboratory, and institute affiliation)

P.I.:	D. LeRoith	Med. Officer, (Res.)	NIDDK/DB
Others:	C.T. Roberts, Jr.	Research Biol.	NIDDK/DB
	H. Werner	Visiting Assoc.	NIDDK/DB
	B. Stannard	Biologist	NIDDK/DB
	M. Adamo	Staff Fellow	NIDDK/DB
	H. Kato	Visiting Fellow	NIDDK/DB

COOPERATING UNITS (if any)

S.E. Mulroney (Georgetown Univ. Wash. D.C.) M. Philippe (Univ. Maryland)
 A. Haramati (Georgetown Univ., Washington, D.C.)

LAB/BRANCH

Diabetes Branch

SECTION

Section of Molecular and Cellular Physiology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892 10/8D48

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES):

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unstructured type. Do not exceed the space provided.)

IGF-I receptors regulate the growth-promoting and differentiative effects of IGF-I and IGF-II. The IGF-I receptor, though encoded by a separate gene, is structurally and functionally related to the insulin receptor, both being transmembrane tyrosine kinases. To investigate structure/function relationships in the IGF-I receptor, we have used a number of different techniques, including (A) isolation and characterization of the proximal promoter for the human and rat IGF-I receptor, genes, (B) mutagenesis of the tyrosine kinase domain of the human IGF-I receptor and (C) analysis of post-receptors events. These studies should contribute to our understanding of the role of IGFs and the IGF receptor in health and disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47002-04 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Gene Expression and Insulin Action

PRINCIPAL INVESTIGATOR (Last, other, professional personnel below the Principal Investigator: (Name, title, laboratory, and specific affiliation)

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unstructured type. Do not exceed the space provided.)

Project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47005 - 20 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Insulin Receptors in Circulating Cells in Man

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	P. Gorden	Director	NIDDK
Others:	C. Hendricks	Biol. Lab Tech.	DB, NIDDK
	E. Collier	Medical Officer	DB, NIDDK
	P. Roach	Clinical Associate	DB, NIDDK
	S.I. Taylor	Chief	DB, NIDDK
	Y. Zick	Visiting Scientist	DB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The present work continues prior investigation of insulin receptors on circulating cells in patients with insulin resistance and diabetes mellitus. Insulin receptors are evaluated for their ability to bind insulin and to act as tyrosine-specific protein kinases. The insulin receptor of a patient with Type A extreme insulin resistance who has normal insulin binding but abnormal tyrosine kinase activity has been sequenced and a mutation found in the extracellular portion of the beta subunit.

We have also showed a strong correlation between the ability of purified anti-insulin receptor antibodies from patients with Type B extreme insulin resistance to inhibit insulin binding and immunoprecipitate the insulin receptor. The correlation of these activities with the biologic activity of the antibodies is not predictable.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47007 17 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Antibodies to Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. S. Taylor Chief, DB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

I N A C T I V E

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 47009-05 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Positron Emission Tomography/NMR Spectroscopy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	R. Eastman	Director	DDEMD, NIDDK
Others:	S. Taylor	Chief	DB, NIDDK
	D. LeRoith	Section Chief	DB, NIDDK
	C. Cochran	RN	CC
	M. Skarulis	Clinical Associate	NIDDK
	B. Koller	Senior Staff Fellow	DB, NIDDK

COOPERATING UNITS (if any)

R. Carson, Nuclear Medicine; C. Bogardis, NIH, Phoenix; Peter Butler, Anton Usala, E. Carolina University; Joseph Frank, Robert Balaban, Diagnostic Radiology, CC; C. Phenekos, Athens, Greece.

LAB/BRANCH

Diabetes Branch

SECTION

Receptors and Hormone Action

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL STAFF YEARS.

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOXES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two protocols have been developed and approved (91-DK-133 and 91-DK-97) to use positron emission tomography (PET) and nuclear magnetic resonance spectroscopy (NMR) to study insulin and IGF-I action in patients with insulin resistance. Both studies use the euglycemic glucose clamp with determination of glucose turnover using the isotope dilution method. PET and NMR are used to study regional hormonal effects in selected tissues such as muscle and fat. We have recently published a case report utilizing this methodology to gain new insights into IGF-II mediated hypoglycemia in a patient with hepatoma. Seven patients (6 insulin resistant, 1 normal) have been entered in the study, and 5 have had euglycemic glucose clamps with insulin and IGF-I. Using NMR spectroscopy it has been possible to isolate the signal from leg glycogen without enrichment with ¹³C-glucose. Studies with PET have not been done to date. One patient with severe insulin resistance and diabetes is receiving IGF-I treatment with benefit, and has been allowed to continue by the FDA. The protocols are on hold by the FDA because a patient had bradycardia leading to asystole while receiving intravenous IGF-I.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47014-21 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the brackets)

Acromegaly and Growth Hormone

PRINCIPAL INVESTIGATOR (Last name, professional personnel below the Principal Investigator; (Name, title, laboratory, and institute affiliation)

COOPERATING UNITS (if any)

LAB BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard procedures (type D) or use extended the space provided.)

This project has been combined with project Z01 DK 47027-06 DB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47018-14 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (20 characters or less. Title may fill one line between the borders.)

Cellular Hormone Like Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. (Name, title, laboratory, and institute affiliation.)

P.I.:	D. LeRoith	Section Chief	DB/NIDDK
	C.T. Roberts	Research Biol.	DB/NIDDK
Others:	M. Adamo	Staff Fellow	DB/NIDDK
	F. Lanau	Guest Worker	DB/NIDDK
	M. McGuiness	Graduate Student	DB/NIDDK

COOPERATING UNITS (If any)

J. Fontana (Univ. Maryland, Baltimore, MD) A. Jaffa (MD Univ. S. Carolina, Charleston, South Carolina)
Carolyn Bondy (NICHD, NIH)

LABORATORY

Diabetes Branch

SECTION

Section of Molecular and Cellular Physiology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892 10/8D48

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Hormone-like peptides including the insulin-like growth factors play important roles in normal growth and development. To further elucidate these roles we have cloned and partially characterized the mammalian IGF-1 genes and studied its expression in physiological and pathological conditions. Thus, we have demonstrated its expression in the nervous system, ovary, kidney and other organs and have found alterations in its expression in pathological conditions, e.g., diabetes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47019 16 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Morphologic Studies of Ligand Binding to Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Phillip Gorden

Director

NIDDK

COOPERATING UNITS (if any)

Institute of Histology and Embryology, University of Geneva School of Medicine, Geneva, Switzerland. (J.L. Carpentier, L. Orci) - Foreign

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL STAFF YEARS

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

This work represents a 16-year collaboration between the Diabetes Branch and the Institute of Histology and Embryology at the University of Geneva. The initial observations demonstrated that polypeptide hormones are taken up by the cell through a process of receptor-mediated endocytosis similar to other biologically important ligands that bind to cells. In the present study, using electron microscopy, we find there is anatomical correlation between the dissociation of 125I-insulin and its localization on the cell surface. Three lines of work using electron microscopy have been followed: (a) We have continued to study the mechanism of receptor-mediated endocytosis. The insulin receptor is a tyrosine kinase, and early studies demonstrate that it is necessary to activate the kinase in order for a ligand to move from the microvillous to the nonvillous coated segment of the cell where it subsequently undergoes internalization; (b) We have continued the study of how ions such as calcium regulate the fusion of intracellular organelles such as endosomes; and (c) the newest studies have involved morphologic localization of newly synthesized receptor proteins from mutant cells and cells that have been mutated at specific glycosylation sites. In summary, all of these studies attempt to relate biochemical studies to specific morphologic correlates.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47022-13 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Receptors in Syndromes of Extreme Insulin Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	S.I. Taylor	Chief, DB, NIDDK	E. Koller	SSF, DB, NIDDK
Others:	A. Cama	Vis. Assoc., DB	E. Wertheimer	Spec. Vol., DB
	D. Accili	Vis. Sci., DB	J. Hone	Clin. Assoc., DB
	C. McKeon	Expert, DB	C.R. Haft	IRTA, DB
	M.A. Lesniak	Chemist, DB	M. Cool	Biotech, DB
	C. Hendricks	Biotech, DB		
	M. Quon	Clin. Assoc., DB		
	P. Gorden	Director, NIDDK		

COOPERATING UNITS (if any)

A.Ullrich- Max Planck Institute, Munich, Germany; T. Kadowaki, University of Tokyo, Japan; H.Kadowaki, Tokyo, Japan; F.Barbetti, Rome, Italy; J. Roth, Johns Hopkins Medical School; M. Muggeo, Verona, Italy.

LAB/BRANCH

Diabetes Branch

SECTION

Receptors and Hormone Action

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS

10.0

PROFESSIONAL

8.0

OTHER:

2.0

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Insulin resistance contributes to the pathogenesis of several disease states including obesity and noninsulin-dependent diabetes mellitus (NIDDM). We have investigated the insulin receptor gene in patients with genetic forms of insulin resistance to gain insight into biochemical defects that give rise to disease. Five classes of mutations have been identified:

1. Impaired receptor biosynthesis, due to either decreased levels of insulin receptor mRNA and/or premature chain termination mutations.
2. Impaired transport of receptors to the plasma membrane, due to missense mutations in the extracellular domain of the receptor.
3. Decreased affinity of insulin binding.
4. Decreased activity of the insulin receptor tyrosine kinase.
5. Accelerated receptor degradation associated with resistance to the effect of acid pH to dissociate insulin from its receptor within the endosome.

Studies are presently underway to estimate the prevalence of mutations in the insulin receptor gene in order to determine whether mutations in this gene contribute to the pathogenesis of the common form of noninsulin-dependent diabetes mellitus.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 47024-13 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biosynthetic Labeling of the Insulin Receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	E. Collier	Medical Officer	DB, NIDDK
Others:	H. Caro	Visiting Fellow	DB, NIDDK
	A. Ohali	Special Vol.	DB, NIDDK
	P. Gorden	Director	NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS

3 women

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

One of the post translational modifications, N-linked glycosylation, of the insulin receptor has been studied in cultured cells using an experimental approach based on mutagenesis of the insulin receptor cDNA at specific sites of potential modification, transfection of the cDNA stably into cultured cells, and then study of these receptors structurally and functionally. Processing of these mutants was investigated by biosynthetic labeling, and isolation of the insulin receptor at various time points. Receptors unable to glycosylate their cells in the first four potential glycosylation sites have abnormal processing. These receptors do not appear on the cell surface and remain in proreceptor form in the endoplasmic reticulum. Mutants of each of these sites are being made in order to determine the significance of each of these sites to the normal processing and intracellular transport of the receptor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 LK 47026- 08 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Tyrosine-specific protein kinase activity associated with the insulin receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. S. Taylor Chief DB, NIDDK

Others: D. Accili Vis. Scientist DB, NIDDK
S. Najjar IRTA DB, NIDDK

COOPERATING UNITS (If any)

University of Catanzaro, Italy (Nicola Perrotti)
Howard University, Washington, DC (D. Seminara)

LAB/BRANCH

Diabetes Branch

SECTION

Receptors and Hormone Action

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS

3

PROFESSIONAL:

2

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In the first step in insulin action, insulin binds to its receptor on the surface of the target cell. The insulin receptor is a transmembrane protein that possesses tyrosine-specific protein kinase activity. When insulin binds to the extracellular domain of the receptor, this activates the receptor tyrosine kinase activity. A growing body of evidence suggests that the activation of the receptor's tyrosine kinase is a necessary step in initiating the biological actions of insulin. Accordingly, we have embarked upon a search for intracellular proteins that are substrates for phosphorylation by the receptor-associated tyrosine kinase. We have identified one such substrate in rat liver plasma membranes: a glycoprotein with an apparent molecular weight of 120,000 (pp 120). In addition to being a substrate for the insulin receptor, pp120 can be phosphorylated by the receptors for epidermal growth factor and insulin-like growth factor I. pp 120 is present in liver from several species, but has not been identified in other tissues. The glycoprotein (pp120) was immunoaffinity-purified using monoclonal antibody HA4. Based on partial amino acid sequence data, pp120 has been tentatively identified as ectoATPase - an enzyme associated with hepatocyte plasma membranes. We have cloned the rat gene encoding pp120/ectoATPase. The gene contains 9 exons, and spans approximately 15 kilobase pairs of DNA. Exon 7 undergoes variable splicing. The transcript lacking exon 7 encodes an isoform in which the cytosolic domain is truncated to only 10 amino acids. The truncation deletes all three putative phosphorylation sites. When the cDNA encoding the full length isoform is expressed by transfection in 3T3 cells, it is capable of being phosphorylated by the insulin receptor tyrosine kinase.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47027-07 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Use of SMS 201 - 995 in Hormone Secreting Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	R. Eastman	Director	DDEMD, NIDDK
Others:	C. Hendricks	Bio Lab Tech.	DB, NIDDK
	P. Gorden	Director	NIDDK
	P. Roach	Clinical Assoc.	DB, NIDDK

COOPERATING UNITS (if any)

B. Weintraub, Chief, MCNEB, NIDDK

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS

2.5

PROFESSIONAL

2.0

OTHER

.05

CHECK APPROPRIATE BOXES.

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This is a project to study the long-term effects of somatostatin analog treatment on acromegaly. Patients are treated unblinded with somatostatin analog as long as it is medically necessary. Treatment and prevention of gallbladder sludge and gallstones, common side effects of somatostatin analogue treatment, is being done with ursodeoxycholate, a bile salt analog that dissolves gallstones. Patients taking somatostatin analogue are treated with ursodeoxycholate if they have gallstones or sludge, and patients without these complications are randomized for placebo controlled treatment with ursodeoxycholate to attempt to prevent sludge formation. Our experience with gall-bladder changes in acromegaly has been recently reported. Five patients have been randomized to masked treatment with ursodeoxycholate, and seven patients have been treated unmasked for sludge or stones. To date no change in pre-existing gallstones or sludge has been seen, and no patient on masked treatment has developed sludge.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47028 - 03 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transcriptional Regulation of the Insulin Receptor Gene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Catherine McKeon	Expert	DB, NIDDK
Assoc. Inv.	Simeon Taylor	Chief	DB, NIDDK
Others:	Gillian Walker	Spec. Vol.	DB, NIDDK
	Hui Chen	Spec. Vol.	DB, NIDDK
	Domenico Accili	Vis. Assoc.	DB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Receptor and Hormone Action Section

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS.

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Regulation of the number of insulin receptors on the cell surface plays a critical role in determining insulin sensitivity. Previously, we have demonstrated that the insulin receptor is regulated at the transcriptional level by glucocorticoids. In order to study the mechanism of transcriptional regulation of the insulin receptor gene, we have cloned the 5' end of the human and mouse insulin receptor gene. We have begun to characterize the proximal promoter and find it has many features of a "housekeeping gene". We have localized a weak enhancer upstream of the promoter that is conserved in both the human and mouse promoters. This enhancer sequence binds nuclear proteins from any difference cell lines. The proximal promoter is probably responsible for the low level expression of the insulin receptor gene which occurs in most cell types. Recently we have identified a region of the first intron which may be involved in tissue specific regulation. This region seems to be responsible for the 10-fold induction of the insulin receptor gene during adipocyte differentiation. Further studies to identify sequences responsible for the transcriptional regulation are underway.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 47029-01 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical modeling of glucose metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	M. Quon	Clinical Associate	DB, NIDDK
Others:	R. Eastman	Director	DDMD, NIDDK
	S.I. Taylor	Chief	DB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Receptors & Hormone Action

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The ability of both insulin and glucose to promote whole body glucose disposal (insulin sensitivity and glucose effectiveness) is important for the regulation of glucose metabolism *in vivo*. Insulin resistance occurs in a variety of pathological states including obesity, non-insulin dependent diabetes mellitus (NIDDM) and hypertension. Decreased glucose effectiveness has been reported in NIDDM. Since insulin and impaired glucose effectiveness may play a role in the pathogenesis of NIDDM, quantitative assessment of these properties is of interest. Recently, a minimal model approach involving mathematical modeling and computer simulation has been widely used to estimate both insulin sensitivity and glucose effectiveness from the results of a single frequently sampled intravenous glucose tolerance test (FSIVGTT). The equations of the minimal model describe changes in plasma glucose concentrations as functions of insulin and glucose concentrations. A computer program uniquely identifies model parameters that generate a best fit to insulin and glucose data obtained during the FSIVGTT. Thus, the minimal model is able to estimate the relative contributions of insulin and glucose to glucose tolerance. We have used the minimal model to generate specific predictions of both insulin independent and insulin dependent glucose metabolism under a variety of conditions. We performed experiments in subjects with insulin dependent diabetes mellitus and in normal subjects to test these predictions. By comparing model predictions with experimental results, we were able to demonstrate that the minimal model underestimates the contributions of insulin and overestimates the contribution of glucose to glucose metabolism. Furthermore, modifications in the FSIVGTT designed to improve minimal model estimates of insulin sensitivity introduced a consistent bias in model estimates of insulin sensitivity. Studies are presently underway to confirm these results and to understand the origin of the discordance between minimal model predictions and experimental results. The data that we collect may allow us to formulate a more accurate and useful mathematical model.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47030-01 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin-receptor related receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	S. Taylor	Chief,	DB,NIDDK
Others:	D. Accili	Visiting Scientist	DB,NIDDK
	H. Jui	Special Volunteer	DB,NIDDK
	Y. Suzuki	Special Volunteer	DB,NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Receptors & Hormone Action

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The insulin receptor is encoded by a single copy gene located on chromosome 19 in the human. In addition, at least two other highly homologous genes have been identified. One gene encodes the receptor for insulin like growth factors (IGF)-I and -II. The third gene in the family encodes another receptor tyrosine kinase. The ligand for this third receptor has not been identified; however, preliminary evidence suggests that this insulin receptor related receptor does not bind insulin, IGF-I, or IGF-II. The goals of this project are two-fold: (1) to identify the ligand for this receptor, and (2) to elucidate the physiological role of the IRR. Toward this end, we have cloned IRR cDNA. By expressing IRR cDNA in cultured cells, we will develop a bioassay for the IRR ligand. In addition, we have cloned the murine IRR gene. A fragment of the cloned gene has been used to construct a targeting vector that is being used to inactivate the IRR gene by homologous recombination in embryonic stem cells. Once this is successful, we will attempt to construct transgenic mice with mutations in the IRR gene. These transgenic mice will be studied in an effort to determine the phenotypic effect of mutations in the IRR gene. This has the potential to provide clues into the physiological roles of the IRR and its ligand.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 47031 - 01 DB												
PERIOD COVERED October 1, 1991 to September 30, 1992														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Regulation of Gene Expression														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P.I.</td> <td style="width: 33%;">M. Reitman</td> <td style="width: 33%;">Senior Staff Fellow</td> <td style="width: 33%;">DB, NIDDK</td> </tr> <tr> <td>Others:</td> <td>L. Abruzzo</td> <td>Clinical Associate</td> <td>DB, NIDDK</td> </tr> <tr> <td></td> <td>Mark Mason</td> <td>IRTA</td> <td>DB, NIDDK</td> </tr> </table>			P.I.	M. Reitman	Senior Staff Fellow	DB, NIDDK	Others:	L. Abruzzo	Clinical Associate	DB, NIDDK		Mark Mason	IRTA	DB, NIDDK
P.I.	M. Reitman	Senior Staff Fellow	DB, NIDDK											
Others:	L. Abruzzo	Clinical Associate	DB, NIDDK											
	Mark Mason	IRTA	DB, NIDDK											
COOPERATING UNITS (if any) G. Felsenfeld, LMB, NIDDK; H. Westphal and E. Lee, LMG, NICHD; J. Grasso, University of Connecticut School of Medicine														
LAB/BRANCH Diabetes Branch														
SECTION Molecular Biology and Gene Regulation														
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland														
TOTAL STAFF YEARS: <div style="text-align: center;">3.0</div>	PROFESSIONAL: <div style="text-align: center;">3.0</div>	OTHER: <div style="text-align: center;">0</div>												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Our goal is to understand further two aspects of gene expression: 1) the mechanisms that operate to 'open' or activate chromatin and 2) those that dictate how a particular gene within an active multi-gene cluster is chosen for expression. The working model evolving from studies of the human β-globin cluster is that control elements upstream of the genes provide a general locus activation function (by an unknown mechanism). These upstream elements also increase the expression of the nearest available genes, with availability determined by the promoter. Human diseases with defects in each of these processes are known (eg. β-thalassemia (Hispanic form) and hereditary persistence of fetal hemoglobin). Knowledge of these topics is part of the background needed for a rational approach to gene therapy.</p> <p>We previously demonstrated that the chicken β^A-globin gene and its 3' enhancer contain information sufficient to guarantee position-independent expression in transgenic mice. (This is quite different from the human β-globin cluster where the β-globin gene needed elements 35 kb to 50 kb upstream to guarantee transgene expression.) In the chick β^A-globin transgenics, the enhancer was essential for transgene expression. Promoter chromatin activation, as measured by DNase I hypersensitivity, correlated with transcription. We have now made mice containing the enhancer without the β^A-globin promoter, and are examining chromatin activation in these mice.</p> <p>We have previously identified pan-erythroid hypersensitive sites upstream of the β-globin cluster. These sites were assayed for enhancer activity, with preliminary data demonstrating such activity for 2 of the sites. We are now characterizing these enhancers with respect to developmental stage specificity, cis elements, and trans factors. We are also examining their function in transgenic mice.</p> <p>A final project, in its early stages, is the production of transgenic mice carrying fragments of chicken genomic DNA containing all 4 chicken β-globin genes, with and without the upstream hypersensitive sites and the β^A/ϵ enhancer. These mice will address the question of which control elements interact with which genes.</p>														

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

701-DK 48001-01 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin-Cell Interaction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

PI: Ian A. Simpson

Visiting Scientist

DB/NIDDK

Others: Samuel W. Cushman

Chief, EDMNS

DB/NIDDK

COOPERATING UNITS (If any)

LAB/BRANCH

Diabetes Branch

SECTION

Experimental Diabetes, Metabolism, and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Inactive.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 48002-01 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin's Regulation of Glucose Transport

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

PI: Samuel W. Cushman Chief, EDMNS DB/NIDDK
Others: I. A. Simpson, Visiting Scientist, DB/NIDDK; O. M. Gonzalez Mulero, Medical Staff Fellow, DB/NIDDK; M. Millo, Special Volunteer, DB/NIDDK; K. I. Timmers, Special Volunteer, DB/NIDDK; C. M. Wilson, IRTA, DB/NIDDK; M. Omatsu-Kanbe, Special Volunteer, DB/NIDDK; T. Ploug, Special Volunteer, DB/NIDDK; J. T. Brozinick, IRTA, DB/NIDDK; D. R. Yver, Chemist, DB/NIDDK; P. A. Ortiz, Special Volunteer, DB/NIDDK; S. Satoh, Visiting Associate, DB/NIDDK; S. J. Vannucci, IPA, DB/NIDDK; M. J. Zarnowski, Biologist, DB/NIDDK; C. Londos, Chief, MRS, LCDB/NIDDK

COOPERATING UNITS (if any)

Receptor, Concord, CA (J. Stagsted, L. Olsson); Department of Biochemistry, University of Bath, Bath, UNITED KINGDOM (G. D. Bolman); Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN (T. Kono); Baker Medical Research Institute, Prahran, Victoria, AUSTRALIA (J. Saltis); Amgen, Thousand Oaks, CA (A. D. Habbelfield).

LAB/BRANCH

Diabetes Branch

SECTION

Experimental Diabetes, Metabolism, and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

8.9

PROFESSIONAL:

8.9

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The possible role of protein kinase C in the regulation of glucose transport in the rat adipose cell has been examined. This study suggests that the mechanisms through which insulin and PMA stimulate glucose transport are distinct but interactive. A new impermeant photoaffinity label has been used for identifying cell surface glucose transporters in isolated rat adipose cells. The results show that while GLUT4 is the major glucose transporter isoform under all conditions, it is selectively and markedly enriched in response to insulin but not PMA which increases GLUT1 and GLUT4 equally. Furthermore, stimulation of glucose transport activity correlates closely with the appearance of GLUT4 on the cell surface in response to both insulin and PMA but does not correlate with the sum of GLUT1 and GLUT4 appearance. The effects of fluorescein isothiocyanate II (FITC) on the actions of insulin in rat adipocytes have been studied. It is concluded that FITC at pH 9.0 (a) renders both glucose transport and phosphodiesterase activities less insulin sensitive presumably by modifying the cellular hormone receptor, and (b) makes glucose transport activity less responsive to insulin presumably by (i) blocking hormone-dependent translocation of glucose transporter and (ii) mildly inhibiting intrinsic glucose transport activity. The subcellular trafficking of tracer-tagged GLUT4 between the plasma membranes and low-density microsomes of rat adipose cells has been studied. Cell-surface GLUT4 have been initially tracer-tagged in the insulin-stimulated state with a $[3H]$ -bis-mannose. The initial experiments show that insulin does not alter the half-time for GLUT4 endocytosis but instead increases the rate of exocytosis. Additional data suggest that the cells' entire complement of GLUT4 is involved in the recycling process. Finally, detailed time-course data suggest that there may be plasma membrane intermediate states in the GLUT4 trafficking pathways. Peptides from the alpha 1 domain of the major histocompatibility complex class I antigen (MHC class I) enhance cellular glucose uptake above that of maximal insulin stimulation, prolong the effect of insulin, and inhibit insulin receptor internalization in rat adipose cells. Based on the new data here, we now propose that MHC class I molecules may be involved in regulation of the internalization process of cell surface integral membrane proteins such as the glucose transporter, IGF-II receptor, and insulin receptor. We have also found that EGF in combination with certain MHC class I-derived peptides is insulinomimetic and that this effect is independent of insulin receptor activity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK48003-01 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in Insulin's Action in Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

PI: Samuel W. Cushman

Chief, EDMS

DB/NIDDK

COOPERATING UNITS (If any)

Diabetes Unit, Beth Israel Hospital, Boston, MA (B. B. Kahn); Department of Medicine, Albert Einstein College of Medicine, Bronx, NY (L. Rossetti); Department of Medicine, University of Texas, San Antonio, TX (R. A. DeFronzo)

LAB/BRANCH

Diabetes Branch

SECTION

Experimental Diabetes, Metabolism, and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Evidence has emerged for a direct role of glucose, independent of insulin, in the regulation of cellular glucose transport and glucose utilization in vivo. In this study, we have investigated potential cellular and molecular mechanisms for this regulatory effect of glucose by determining how normalization of glycemia without insulin therapy in diabetic rats influences 3-O-methylglucose transport and the expression and translocation of two genetically distinct species of glucose transporters (GTs) in adipose cells. The data show that normalization of blood glucose in diabetic rats with phlorizin, which impairs renal tubular glucose reabsorption and thus enhances glucose excretion, restores insulin-stimulated glucose transport in adipose cells and insulin-mediated glucose disposal in vivo and thus that ambient glucose independent of ambient insulin can regulate the glucose transport response to insulin in isolated adipose cells and changes in responsiveness parallel alterations in in vivo glucose uptake. Since this effect can occur without alteration in the expression of the two species of glucose transporters present in adipose cells or in their translocation to the plasma membrane in response to insulin, it may result from changes in GT functional activity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK48004-01 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin's Regulation of Hormone Binding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

PI: Samuel W. Cushman

Chief, EDMNS

DB/NIDDK

Others: Ian A. Simpson

Visiting Scientist

DB/NIDDK

COOPERATING UNITS (If any)

LAB/BRANCH

Diabetes Branch

SECTION

Experimental Diabetes, Metabolism, and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Inactive.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01-DK-48005-01 DB
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT <u>(80 characters or less. Title must fit on one line between the borders.)</u> Counterregulation of Insulin's Action by Catecholamines		
PRINCIPAL INVESTIGATOR <u>(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)</u>		
P.I. Ian A. Simpson, Ph.D. Haruo Nishimura, M.D., Ph.D. Susan J. Vannucci, Ph.D.	Visiting Scientist Visiting Fellow I.P.A (Intergovernmental Personnel Agreement),	EDMNS/DB/NIDDK EDMNS/DB/NIDDK EDMNS/DB/NIDDK
Samuel W. Cushman, Ph.D. Mary Jane Zarnowski	Chief Biologist	EDMNS/DB/NIDDK EDMNS/DB/NIDDK
COOPERATING UNITS <u>(If any)</u> C. Londos, Chief, MRS/LCDB/NIDDK G. D. Holman, Department of Biochemistry, University of Bath Claverton Down, UNITED KINGDOM		
LAB/BRANCH Diabetes Branch		
SECTION Experimental Diabetes, Metabolism, and Nutrition Section		
INSTITUTE AND LOCATION NIDDK, NIH, Building 10, Room 5N102, Bethesda, MD 20892		
TOTAL STAFF YEARS: 2.1	PROFESSIONAL: 1.7	OTHER: 0.4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK <u>(Use standard unreduced type. Do not exceed the space provided.)</u> <p> Earlier reports from our laboratory demonstrated that hormones which activated adenylyl cyclase (catecholamines, glucagon, ACTH) in adipose cells inhibited insulin-stimulated glucose transport activity. Conversely hormones that inhibited adenylyl cyclase (adenosine, PGE1, nicotinic acid) activated transport activity. In contrast to insulin, these hormones did not alter the subcellular localization of either GLUT1 or GLUT4, but instead appeared to modulate transport activity at the level of plasma membrane. </p> <p> Recently we have investigated whether these changes in transport activity could be attributed to changes in the phosphorylation state of GLUT4. A partial phosphorylation of GLUT4 (0.1-0.2 mol/mol) observed in basal cells was unaltered by insulin but was slightly increased by isoproterenol. However this increased phosphorylation was confined to those transporters residing in the intracellular membranes. Furthermore it was observed both in the presence and absence of adenosine receptor activation whereas transport inhibition was only detected in the absence of adenosine agonists, suggesting that transporter phosphorylation does not explain the changes in transport activity. </p> <p> An alternative approach to answer these questions has involved the use of an impermeant [3H]-bis-mannose photolabel to monitor hormonally induced changes in accessibility of the glucose transporters in intact adipose cells. The inhibition of glucose transport activity induced by isoproterenol correlated closely with the decrease in GLUT4 accessibility. Isoproterenol appeared to induce a change in the conformation of the GLUT4 transporter such that it cannot either bind photolabel or transport glucose. Adenosine prevented these effects and rendered the transporter more accessible. </p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 48006-01 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in Insulin's Action with Fasting/Refeeding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

PI: Samuel W. Cushman Chief, EDMNS DB/NIDDK

Others: Ian A. Simpson Visiting Scientist DB/NIDDK

COOPERATING UNITS (If any)

LAB/BRANCH

Diabetes Branch

SECTION

Experimental Diabetes, Metabolism, and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Inactive.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 48007-01 DB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glucose Transport in Mammalian Brain

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

P.I. Ian A. Simpson, Ph.D.	Visiting Scientist	EDMNS/DB/NIDDK
Koteswara Chundu, M.D.	Special Volunteer	EDMNS/DB/NIDDK
Frances Maher, Ph.D.	Visiting Fellow	EDMNS/DB/NIDDK
Susan J. Vannucci, Ph.D., I.P.A. (Intergovernmental Personnel Agreement)		EDMNS/DB/NIDDK
Lynn Sorbara, Ph.D.	Staff Fellow	EDMNS/DB/NIDDK
Theresa Davies-Hill	Biologist	EDMNS/DB/NIDDK

COOPERATING UNITS (If any)

Dr. Peter Davies	Albert Einstein College of Medicine	Pathology Department
Dr. Kenneth Seamon	CBER/DBB/FDA	

LAB/BRANCH

Diabetes Branch

SECTION

Experimental Diabetes, Metabolism, and Nutrition Section,

INSTITUTE AND LOCATION

NIDDK, NIH, Building 10, Room 5N102, Bethesda MD 20892

TOTAL STAFF YEARS:

4.4

PROFESSIONAL:

3.1

OTHER:

1.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study represents a major new direction in my laboratory to investigate the regulatory mechanisms involved in the transport of glucose in mammalian brain. Two glucose transporter isoforms, GLUT1 and GLUT3, have been identified in brain. In whole brain GLUT1 is detected as two molecular weight forms: a 55kDa form which is concentrated in the endothelial cells of the blood-brain barrier and a 45 kDa form present in vascular-free cortical membranes, as well as in primary cultures of neurons and glia. In the rat, GLUT3 is expressed exclusively in the brain but is notably absent from the blood-brain barrier of both rat and human. Ontogeny studies indicate that in the rat brain, the concentration of GLUT3 and both forms of GLUT1 progressively increase from the fetus through 30 days postnatally. We have detected GLUT3 in adult rat brain in all regions except the adenohypophysis and pineal gland, in primary neuronal but not astrocytic cultures, and in cultured cells of neuronal origin, e.g. PG12 and NG108-15 cells. Studies with primary cultures revealed an increase in the GLUT 1 and 3 mRNA and protein levels which peaked at 4 and 6 days respectively, coincident with maximal glucose transport capacity and complete cellular differentiation. Quantitative studies using either a specific [³H]-bis-mannose photolabel or [³⁵S] methionine incorporation indicated that the concentration of GLUT3 is five times greater than GLUT1 in these cultured neurons. In serum-free cultures GLUT1 and GLUT3 expression appears to be unaffected by ambient glucose concentrations but is reduced by lowering the potassium concentrations.

ANNUAL REPORT OF THE CLINICAL HEMATOLOGY BRANCH

National Institute of Diabetes and Digestive and Kidney Diseases

Study of Immunology of Blood Cell Deficiencies

Objectives:

To study the immunochemistry of allergic disorders affecting blood cells and effects of the immune reactions on cellular physiology, biochemistry, and in vivo cellular kinetics.

Methods employed:

Isoelectric focusing; anion exchange chromatography; enzyme activity assays; gel filtration of platelets; platelet activation by thrombin and other agonists; Western blotting; PVDF blotting of proteins for sequencing; microtiter enzyme-linked immunoassays for Ig quantitation; fluorescence-activated cell scanning; purification of antibodies by adsorption/elution; monoclonal antibody antigen capture assays; fibrinogen binding by platelets; complement fixation; electron microscopy; native gel electrophoresis; enzyme (calpain) purification from platelets; cryoprecipitation; lectin and monoclonal affinity chromatography for glycoprotein purification; immunoprecipitation; monocyte elutriation; protein and RNA synthesis using labeled precursors; specific mRNA assays for IL6, IL1, and TNF; intracellular Ca^{++} flux measurements; HPLC analysis of arachidonate metabolites, prostaglandins, leukotrienes, and HETEs; platelet serotonin and ATP secretion studies; PCR amplification of modified platelet GP cDNA and expression of GPs for studies of autoAb epitope specificities.

1. Multiple Ig Classes, Specificities, and Affinities of Anti-glycoprotein Autoantibodies in Sera of Patients with Chronic Idiopathic Thrombocytopenic Purpura (ITP) and Other Immune Thrombocytopenias.

Findings:

Plasma antiplatelet autoantibodies (autoAbs) responsible for ITP decrease recovery and survival of homologous platelets in almost all thrombocytopenic patients, yet recent reports on serologic studies emphasizing reactions of IgG autoAbs with platelet glycoproteins (GPs) indicate that >50% of ITP plasmas contain either no detectable Abs or Abs that react primarily with presumptive cytoplasmic domains of membrane GPs. We modified existing immunobead techniques to increase sensitivity by using purified GPs and found anti-GPs in sera of 85% of 47 chronic ITP patients. IgA and IgG Abs were each present in 68% of patients, together in 51%; and IgM agglutinins were present in 15%, always with another Ab class. GPs Ib/IX, IIb/IIIa, IV, and Ia/IIa were targets in 83%, 81%, 38%, and 30% of cases, respectively; 93% of positive sera reacted with more than one GP, and GP IV or Ia/IIa

was never the sole target. For maximum positive results (85%), tests for IgA and IgG Abs against GPs Ib/IX and IIb/IIIa were necessary and sufficient. As judged by adsorptions with solid phase C-terminal dodecapeptides of GP IIIa and Ib α and adsorptions with intact platelets, Abs against C-terminal epitopes were found in 66% of cases, accompanied by Abs against other epitopes of the same or a different GP in all cases. Sera of 40% of 16 patients recovering from posttransfusion purpura and drug purpura had autoAbs against only internal GP epitopes, and sera from 15 patients with decreased platelet production had no anti-GPs, suggesting that Abs against internal GP epitopes are a secondary phenomenon of platelet destruction. Different ITP Abs present in excess bound to GP-coated beads in amounts lower than known high-affinity alloantibodies against the same GPs, indicating that most ITP Abs are of relatively low affinity and therefore require highly sensitive methods for their detection. Although none of these diverse serologic observations correlated with severity or remission of ITP, Abs against external GP epitopes were limited to ITP sera.

Planned Work

a. We will attempt to identify amino acid sequences of the specific epitopes in GPs IIIa and Ib that appear to be the targets of pathogenic autoAbs in ITP by using site-directed mutagenesis of the cDNA coding for these GPs, PCR amplification, and a bacterial expression system. GP segments will be screened by Western blots using patients' Abs we have already identified in the above study.

b. Approximately 20% of patients refractory to platelet transfusions have serum antibodies against platelet-specific antigens which do not correspond to known platelet allotypes. We will test a series of refractory patients for antibodies against C-terminal cytoplasmic epitopes in addition to alloantigenic external epitopes of major platelet GPs to help clarify obscure serology in these cases.

c. We will prepare purified ITP Abs by elution from ITP platelets and from normal platelet membranes incubated in ITP sera to determine affinities of their reactivities when exposed to known quantities of the various major platelet GPs immobilized on polystyrene beads. This information may help explain poor correlations between serologic observations and the course of ITP, and the controversial significance of platelet-associated immunoglobulins in this disease.

d. Purified immune globulin (IVIgG) is effective therapy in a high percentage of patients with ITP, but reasons for this are obscure. The effects of IVIgG on reactions of patients' antibody with specific platelet GPs will be evaluated, particularly with respect to possible identification of anti-idiotypic Abs.

e. We will characterize anti-GPs in HIV-associated thrombocytopenia as in b above. Whether Abs are responsible for thrombocytopenia in HIV-infected patients, and, if so, whether they are similar to those in ITP or directed against viral components are open questions.

2. Pathophysiology of Posttransfusion Purpura (PTP).

Findings:

PTP is a disease almost exclusively in women caused by alloantibodies against transfused platelet antigens. In the initial report defining PTP, we proposed as a mechanism of thrombocytopenia, that foreign antigen (PlA¹ in a PlA¹-negative patient who in years past has given birth to a PlA¹-positive child) survives in vivo longer than the period of anamnestic antibody induction, and that antibody complexed with foreign antigen, is adsorbed by autologous platelets causing their destruction. We reported finding nonsedimentable microparticulate platelet-derived antigen (PlA¹ antigen) in stored blood and plasma that could be adsorbed in vitro by PlA¹-negative platelets in the presence or absence of anti-PlA¹ antibody. Last year we found that, during periods of thrombocytopenia, plasmas from 6 of 6 PlA¹-negative PTP patients contained microparticulate PlA¹ antigen derived from platelets transfused up to 10 days previously. Antigens survived in quantities sufficient to coat autologous antigen-free platelets with 200 to 1000 antigen-antibody complexes per platelet which is sufficient to cause thrombocytopenia under experimental conditions in vivo. Of special significance was the finding that the syndrome could be reproduced in guinea pigs by infusing nonsedimentable platelet microparticles obtained from human PlA¹-positive blood followed by infusion of affinity purified anti-PlA¹ from patients with PTP. Guinea pigs developed thrombocytopenia only when antigen was followed by antibody or when antigen-antibody complex were infused, but not when antigen or antibody was infused alone. Platelet microparticles that were adsorbed contained the activation antigen, GMP-140, cytoplasmic carboxy-terminal epitopes of platelet membrane glycoproteins IIb/IIIa and Ib/IX, fibrinogen, and fibronectin, indicating that the particles were released from activated platelets. Platelet activation could be associated with storage under blood bank conditions or with senescence of transfused platelets in vivo. Cooling to 4°C under blood bank conditions caused elevation of platelet cytoplasmic calcium and tyrosine phosphorylation of the 130 kD protein associated with platelet activation (see project Z01 DK51, 001-32CHB) which is known to produce platelet microvesiculation. In electron microscopic studies so far we have identified nonsedimentable antigen-containing vesicular structures of 400 to 4000 angstroms in diameter concentrated in lipid-rich fractions of normal plasma from

cooled and stored blood. These structures are not associated with lipid spheres of LDL.

Planned work:

a. To exploit the guinea pig model to determine stoichiometry, complement dependence, and other attributes of immune reactions that result in platelet destruction and to define the smallest polypeptide chain of GP IIIa containing the Pl^{A1} epitope that will support immune reactions causing thrombocytopenia.

b. To determine whether there are increases in microparticulate material in plasma from patients with destructive thrombocytopenias and whether adsorption of this material with antigenic cytoplasmic or cryptic epitopes oriented externally (see study #1 above) may contribute to platelet destruction.

c. To continue development of gold and biotin-streptavidin labelling techniques for characterizing microparticles by electron microscopy.

3. Pathophysiology of Idiopathic Thrombocytopenic Purpura.

Findings:

Macrophages appear to participate in destruction of platelets in ITP as evidenced by return of platelets in the face of persistent circulating antibody during therapeutic responses to splenectomy and adrenocorticosteroids. There are several reports indicating that platelets form rosettes or clusters with monocytes in the presence of ITP plasma, suggesting monocyte activation by an immunologic reaction. However, these observations require subjective interpretation, are quite variable, and may be artifacts of nonimmune platelet activation which causes platelet-monocyte interaction. We found no differences in effects of normal and ITP plasmas on platelet-monocyte rosetting. In attempts to objectively evaluate effects of anti-platelet antibodies on monocyte activation in the presence of platelets, elutriated monocytes were sensitized (armed) with 95% pure IgG derived from ITP plasma or plasma from patients with high-titer alloantibodies (anti-Pl^{A1}) due to PTP (see above). The armed monocytes were loaded with ³H-arachidonic acid (AA) and exposed to platelets adherent to tissue culture wells. AA metabolites secreted in culture supernatants were analyzed by HPLC. Last year, on the basis of analyses with 4 ITP and 3 PTP patients, it appeared that leukotriene (LT) and hydroxyeicosanoic acid (HETE) metabolites were consistently higher than control values obtained with normal IgG; but after doubling the number of determinations to 14 patients and 14 controls during the past year, an overlap between the two groups was apparent, and significant differences remained only for LTB₄ with ITP Abs (p<.01) and PTP Abs (p<.05). It appears that adherence of platelets to wells causes exposure of

the GMP-140 platelet antigen which initiates monocyte activation in control assays and obscures effects that might be caused by specific antibodies.

Planned work:

a. If lymphokines stimulate platelets, activation of monocytes by platelet-antibody systems might contribute to platelet destruction in the RES by promoting platelet secretion and aggregation. There is no information on the direct effects that various cytokines might have platelet function. This will be tested.

DEPARTMENT OF HEALTH AND HUMAN SERVICES-PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 51, 007-34 CHB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Immunology of Blood Cell Deficiencies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Raphael Shulman	Chief	CHB:NIDDK
Others:	Diane M. Reid	Staff Fellow	CHB:NIDDK
	Charles E. Jones	Chemist	CHB:NIDDK
	R.-Y. He	Visiting Fellow	CHB:NIDDK
	William Jackson	Research Fellow	CHB:NIDDK

Cooperating Unit (If any) Charles Carter (CC-DTM); Anna Husebekk, University Hospital of Tromsø Norway; Ileana Lopez, Yale University, J. Blanchette-Mackie (LCDE - NIDDK); Timothy Mauge, University of Maryland

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.25

PROFESSIONAL:

2.75

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human Subjects ☒ (b) Human Tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Immune-mediated thrombocytopenias are the leading cause of hemorrhagic diathesis, the prevalence being greater than 1 per 2,000. Autoimmune idiopathic thrombocytopenic purpura (ITP) is the most common type. Sensitizing antigens (Ags) in ITP have not been defined and most serologic tests for antiplatelet antibodies (Abs) have been insensitive or nonspecific. We developed an assay for Abs against major platelet membrane glycoproteins (GPs) and their peptide derivatives which detected Abs in 85% of 47 ITP patients, defined the classes and affinities of Abs, and determined percentage of cytoplasmic versus external membrane epitopes involved in sensitization.

Posttransfusion purpura (PTP) is a rare disorder occurring 5-7 days after transfusions containing platelet material. Patients' plasmas contain an alloAb against a platelet Ag lacking in the patient but present in transfused blood. Reasons for destruction of autologous platelets have been obscure. We have now shown that there are platelet-derived membrane Ags in normal donor plasma that, when transfused, elicit alloAbs that adsorb to recipients' platelets as Ag-Ab complexes and cause platelet destruction. We developed a guinea pig model to reproduce this disease and have used electron microscopy to identify the platelet microparticles.

Occurrence of phagocytosis in immune platelet destruction is controversial. We studied metabolic responses of monocytes armed with antiplatelet auto- and allo-Abs and exposed to appropriate platelet Ags for indications that monocytes may participate in immune platelet destruction. Arachidonate metabolites including thromboxanes, leukotrienes, and prostaglandin E2 were increased in monocytes armed with antibodies from ITP or PTP patients and then exposed to platelet-specific Ags, suggesting that cellular immunity plays a role in immune thrombocytopenia.

Among patients referred with platelet dysfunction, 3 had unique Abs causing hemorrhage by interfering with the RGD binding site of the integrin, GPIIb/IIIa. A patient with drug hemolytic anemia had antibodies reactive with band 3 which is the first time a specific red cell membrane protein has been implicated in this syndrome.

DEPARTMENT OF HEALTH AND HUMAN SERVICES-PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 51, 001-34 CHB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT(80 characters or less. Title must fit on one line between the borders.)

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

PRINCIPAL INVESTIGATOR(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Raphael Shulman	Chief	CHB:NIDDK
Others:	William Jackson	Research Fellow	CHB:NIDDK
	Charles E. Jones	Chemist	CHB:NIDDK
	Diane M. Reid	Staff Fellow	CHB:NIDDK

COOPERATING UNITS(IF ANY) Charles Carter (CC-DTM)

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.75

PROFESSIONAL:

1.25

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

<input checked="" type="checkbox"/> (a) Human Subjects	<input checked="" type="checkbox"/> (b) Human Tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

SUMMARY OF WORK(Use standard unreduced type. Do not exceed the space provided.)

Platelets prevent hemorrhage by a secretory process controlled through an intracellular chain of biochemical events similar to that in all other secretory cells. Tyrosine kinase (TK) activity, which causes tyrosine phosphorylation (TP) of specific cellular proteins is temporally associated with secretion by platelets and other cells, as well as with cellular responses such as growth, contact inhibition, and malignant transformation. The role of TP in these processes is not known. Last year, we found increased platelet cytoplasmic calcium that accompanies secretion promotes TP of a 130 kD major cytoplasmic protein, while a homeostatic level of calcium in storage compartments promotes tyrosine dephosphorylation of this protein. (J Biol Chem 226:16911-16916, 1991). This year, we identified the 130 kD platelet protein as vinculin by its reaction with both monoclonal anti-phosphotyrosine and anti-vinculin on Western blot, by affinity chromatography isolation with both antibodies, and by isoelectric focusing of a single protein with a pI for vinculin that reacted with anti-vinculin and anti-phosphotyrosine. Since TP of vinculin is under control of cytosolic and stored calcium and vinculin is known to be linked via α -actinin to platelet glycoprotein IIb/IIIa which mediates calcium influx, vinculin may be involved in regulation of platelet membrane calcium channels.

ANNUAL REPORT OF THE GENETICS AND BIOCHEMISTRY BRANCH

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Biochemical Genetics Section

Dr. Proia and his colleagues have continued their studies of the lysosomal enzyme β -hexosaminidase (a deficiency of which is responsible of Tay-Sachs disease). This enzyme is made up of two subunits α and β . Over the last few years they have cloned the cDNAs and genes for both chains of the human β -hexoaminidase. In the last year they have started to investigate the mechanism by which the major mutation in Ashkenazi Jewish Tay-Sachs carriers and patients, a 4 base pair insertion in exon 11 of the HEXA gene, leads to the degradation of the mRNA for this gene. They have developed a system to investigate the effect of the 4 bp on HEXA mRNA expression. Cells transfected with a mini-gene containing the 4 bp mutation, however, yielded undetectable HEXA mRNA. The mRNA level was restored with a mini-gene containing the 4 bp mutation when 1 bp was deleted upstream to correct the reading frame.

In addition, Dr. Proia and his colleagues have started to examine how in particular tissues and under certain circumstances the expression of lysosomal enzymes is enhanced over the constitutive levels typical for genes encoding house keeping enzymes. To that end they have characterized the promoter region of the HEXB gene. They have found that both the mouse and human HEXB genes contain a homologous 50 bp region with functional promoter activity within 40 bp of the respective RNA start sites. The regions contains several binding sites for transcription factors.

Molecular Genetics Section

Dr. Ackerman and collaborators have continued their work on the mode of action of the *Aspergillus* toxin α -sarcin and other substances that inhibit protein synthesis. They had previously demonstrated that several toxins all inactivate protein synthesis in cells by attacking a highly conserved sequence in 28S ribosomal RNA. They had also shown that modified oligonucleotides and ribozymes complementary to the same region of 28S RNA also abolish protein synthesis. They have now demonstrated that certain nucleases are surprisingly efficient in abolishing protein synthesis and that one of these nucleases selectively hydrolyzes tRNA when injected into *Xenopus* oocytes.

On a different project Dr. Ackerman and his colleagues have extended their studies on DNA repair. They have demonstrated pyrimidine dimer repair by micro-injecting UV-irradiated DNAs into *Xenopus* oocytes or in a cell-free system by using an extract derived from oocytes. They have also studied DNA repair for alkylated and chemically modified DNA as well as DNA replication with their repair extract.

Dr. Hsieh and her colleagues are examining the effects of mismatched bases on DNA branch migration. When heteroduplex DNA is formed in the course of the recombination or repair of DNA the heteroduplex regions can undergo branch migration. Depending on the direction of this branch migration, the length of the heteroduplex region will increase or decrease. Longer heteroduplex regions will result in an increase in the

amount of genetic information that is exchanged between homologues. In order to study this problem they have developed a model four stranded system to systematically examine the effect of mispaired or unpaired bases on nonenzymatic branch migration.

Using a series of short duplexes 40-60 bp long, they have observed that the reaction consists of several distinct steps, annealing of the two duplexes, initiation of a Holliday junction and branch migration. Their results demonstrate that a single mismatched base pair impeded branch migration. Their work also indicates that the block on branch migration imposed by mismatches is more pronounced in the presence of Mg^{2+} .

Dr. Hsieh and coworkers are also attempting to obtain a chicken cDNA clone for a type I DNA ligase. They wish to exploit the fact that chicken B cells carry out homologous recombination at unusually high frequencies to create a ligase-deficient chicken B cell line by gene targeting. In this manner, they may be able to assess the physiological function of type I ligase in vertebrate cells.

Dr. Camerini-Otero and his colleagues continue their studies aimed at dissecting the biochemical steps involved in genetic recombination. Over the last few years they have focused on identifying the proteins responsible and how they work. They have chosen to concentrate their efforts on a key early step: strand exchange between homologous parental DNAs. *In vitro*, the product of this exchange is joint molecule composed of a single-strand DNA joined to the end of a duplex DNA. First they identified several eukaryotic proteins responsible for this reaction. Recently they have established a new paradigm of this homologous pairing: that all recombinases, including the *E. coli* recA protein, can hybridize a single strand of any sequence and an intact duplex. That is, the three strand for a novel DNA triplex (which they have designated R-form DNA) in which the third strand may include both purines and pyrimidines. Recently, they have used thermal denaturation of chemically substituted DNAs and chemical footprinting of R-form DNA to confirm that the third strand in R-form DNA is in the major groove of the duplex.

Dr. Camerini-Otero and colleagues have also been able to isolate synaptic complexes consisting of all the three strands and recA. These structures have been studied in detail and will continue to be the basis of additional structural investigations. The kinetics of formation of these complexes are being used as a model for the homology search process in the obligatory recombination events during meiosis. The synaptic complexes have also been used to develop a method for the selective cleavage of human DNA (RecA-Assisted Restriction Endonuclease (RARE) cleavage); this method is now being applied to map and clone large fragments of DNA close to the Huntington Disease and Multiple Endocrine Neoplasia 1 genes.

In addition, Dr. Camerini-Otero and colleagues have been successful in cloning the gene for a thermostable recA homologue. This protein should be very useful for mechanistic studies and for several important applications in biotechnology. Finally, they have cloned from yeast and higher eukaryotes several sequence homologues to recA. They are attempting to establish functional homology between the proteins encoded by these genes and the *E. coli* recA protein.

Mechanisms of Gene Regulation Section

Investigators in this section conduct basic scientific research on the mechanisms of action of the thyroid hormone and effects of a family of POU-domain genes on anteroposterior axis formation during amphibian embryogenesis.

Dr. Nikodem and colleagues continue to study how the rate of transcription of target genes is modulated by binding of thyroid hormone receptor-hormone complex to the specific DNA. The thyroid hormone receptor α gene is alternatively spliced to give the α receptor and a variant which does not bind thyroid hormone. An additional α like receptor, Rev-erbA α , is transcribed from the opposite strand downstream of thyroid hormone receptor α but convergent over 256 bases with the variant and not the α receptor. The overlapping regions of these two transcripts raises the possibility that a sense-antisense hybrid might form *in vivo* and thus could modulate a level of the variant with concomitant increase in production of the α mRNA. No functional results could be seen *in vivo* from potential anti-sense pairing of Rev-erbA α with the variant, since the level of variant mRNA stays high from fetal to adult stage, while the Rev-erbA α is present only in adult tissues.

An additional means in controlling the action of thyroid hormone could be provided by regulating expression of the receptor gene. Dr. Nikodem and her colleagues cloned and sequenced the 5' flanking region of the receptor α and minimal promoter sequences required for expression of this gene were characterized. This promoter is GC rich and it does not contain either a TATA or CAAT box. Furthermore the promoter activity is down regulated by the receptor α itself in hormone dependent fashion.

In another study Dr. Nikodem's group fully characterized two thyroid hormone response elements. Malic enzyme element functions as a thyroid hormone-receptor dependent enhancer of the GC rich malic enzyme promoter and the myelin basic protein element as an enhancer of the promoter containing TATA and CAAT boxes. There are thyroid hormone dependent quantitative differences in transcriptional activation with the receptor α and β on these two thyroid hormone response elements. So far, they demonstrated that both the myelin basic protein thyroid hormone response element (arranged as an inverted palindrome) and a TATA like sequence are required for more efficient thyroid hormone responsiveness elicited by the β receptors. In the case of the malic enzyme promoter, more efficient thyroid hormone dependent activation by the α receptor requires the malic enzyme element (arranged as a direct repeat) and an element within 122 nucleotides upstream from the start of transcription.

Dr. Nikodem and collaborators also constructed plasmids expressing the receptor α with polyhistidine tail. Baculovirus and E. coli expression systems and a nickle column have been used to purify the overexpressed receptor. This receptor is being used to study various parameters in heterodimer formation of the receptor and other nuclear proteins and effect of the ligands in binding of these complexes to thyroid hormone response elements.

Dr. Sato and her colleagues are investigating functions of the POU domain gene family which has been shown to be important in tissue specific gene regulation during development. It has been proposed that POU class III transcription factors act in a combinatorial fashion to establish various neuronal phenotypes in the brain. The role of two of these POU class III factors, XLPOU 1 and XLPOU 2, in early neural development was studied. *In situ* hybridization analysis of *Xenopus* embryos has demonstrated that in the neural plate, XLPOU 1 gene expression is restricted to the future midbrain and hindbrain. In tailbud stage embryos, additional XLPOU 1 gene expression is observed in the forebrain and eyes. XLPOU 2 gene expression is observed in the ventral forebrain, midbrain, and hindbrain. XLPOU 1 and XLPOU 2 should prove to be useful markers in studying how the anterior part of the brain is established. When embryos are treated with retinoic acid or its derivatives, anterior-posterior polarity in the nervous system is disrupted, resulting in a posteriorization of the embryonic brain. Using lineage tracers to map the cell fate of anterior blastomeres, it was demonstrated that the underlying

mechanism of a retinoic acid posteriorization of the brain was due to a change in cell fate. Furthermore, concurrent with the loss of forebrain structures that occurs with low doses of retinoic acid, cells in a more posterior position, the X1POU 1- and X1POU 2- expressing cells of the midbrain and eye, were shown to proliferate.

Endocrinology Section

Studies are continuing on the diagnosis of thyroid cancer and its treatment with I-131. Twenty patients with high-risk cancer have been entered into the protocol using doxorubicin (adriamycin) as a radiation enhancer. Seven patients were randomized to receive the combination therapy and a total of 19 such treatments have been administered with no apparent additional toxicity.

A second group of patients were given recombinant human TSH to stimulate I-131 uptake in thyroid remnants following thyroidectomy. This Phase I/II study is part of a multi-institutional effort in collaboration with Dr. Bruce Weintraub and the Genzyme Corporation. Initial results indicate that the drug appears to be effective and safe; the next phase will be to test rhTSH in patients with metastatic cancer. If effective, it will greatly simplify the diagnosis and treatment of metastatic thyroid cancer by avoiding the need for withdrawal of thyroid hormone and inducing hypothyroidism.

A third group of patients is under study to evaluate serum thyroglobulin (TG) as a guide to I-131 therapy when the diagnostic whole body I-131 scan is negative. In 14 patients who received up to 3 treatments with 150 to 300 mCi the post therapy scan was positive in 13 patients (21 of 28 treatments) and 7 showed metastatic disease. Serum TG decreased in 82% of patients and reverted to $\leq 5 \mu\text{g/ml}$ in 29%; the post therapy scan became negative in 50%. Further follow-up is required to determine whether this therapeutic effect will improve prognosis and survival.

Luigi Bartalena continued his study of the effect of interleukin-6 (IL-6) on the synthesis of thyroxine and cortisol binding globulin (TBG and CBG) in cultured human hepatoma cells (HepG2). These serum transport proteins are members of the serine antiprotease (serpin) family, which includes α_1 -antitrypsin and α_1 -antichymotrypsin, "acute phase proteins" whose synthesis increases dramatically during infection, trauma, etc. Both TBG and CBG synthesis and secretion were decreased by IL-6. Whereas transcription of the TBG gene was decreased, no change in transcription of the CBG gene was detected. This suggests divergence of a common ancestral gene for these proteins. The decrease in CBG in the acute phase phenomenon could result in an increase in local free cortisol which might play a role in combating inflammation.

Salvatore Benvenga and Hans Cahnmann continued their analysis of thyroxine (T_4) binding to high-density lipoproteins (HDL) by photoaffinity labeling. In addition to the previously-demonstrated labeling of apoA-1, the major HDL apolipoprotein, T_4 was shown to interact with several additional apolipoproteins, including apoA-II, apoA-IV, apoC-1, II and III, and apoE. Previous experiments showed that the T_4 bound to LDL can enter fibroblasts through interaction with LDL (apoB-100) plasma membrane receptors. The possibility that binding of T_4 to the apolipoproteins of HDL may affect the uptake or release of T_4 from fibroblasts and hepatocytes and the intracellular targeting of the hormone will be explored.

Mark Lakshmanan, now at Case Western Reserve University, continued his work on the effect of amino acids on the receptor-mediated entry of T_4 into neuroblastoma cells (NB41A3). It was previously shown that phenylalanine is a competitive inhibitor of T_4

uptake, and Lakshmanan has now demonstrated that phenylalanine uptake by NB41A3 cells is inhibited by T_4 . Although the high concentration of T_4 required for this effect indicates that this is unlikely to be physiologically important, it is quite possible that inhibition of T_4 uptake by phenylalanine may have a role in the pathophysiology of phenylketonuria.

Hans Cahnmann has worked to improve the synthesis of N-bromoacetyl-3, 3',5-triiodothyronine and N-bromoacetyl thyroxine. These compounds are widely used for affinity labeling of thyroid hormone binding proteins, but previous methods gave impure preparations of uncertain identity. Methods have now been perfected for the production of the pure reagents, both in macro and micro amounts, the latter being required for the synthesis of radioiodine-labeled compounds used in affinity labeling. The purification procedure utilized high-speed countercurrent chromatography, in collaboration with Yochiro Ito (NHLBI).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 52008-13 GBB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gene Expression and Human Genetics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R.D. Camerini-Otero	Chief	GBB, NIDDK
Others:	R. Gardner	Clinical Associate	L. Sastry
	E. Angov	IRTA Fellow	J. Yancey-Wrona
	M. Kim	Visiting Fellow	G. Poy
	L. Ferrin	Research Associate	L. Pike
	S. Pati	IRTA Fellow	
			Visiting Associate
			IRTA Fellow
			Biologist
			Biologist

COOPERATING UNITS (if any)

Carol Camerini-Otero - Diabetes Branch, NIDDK
Victor Zhurkin and Robert Jernigan - Laboratory of Mathematical Biology, NCI

LAB/BRANCH
Genetics and Biochemistry Branch

SECTION
Molecular Genetics Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS
10.00

PROFESSIONAL:
9.5

OTHER:
0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to dissect the biochemical steps involved in genetic recombination we have chosen to focus on a key early step: strand exchange between homologous parental DNAs. *In vitro*, the product of this strand exchange reaction is a joint molecule composed a single-strand DNA joined to one end of a linear duplex DNA. We have established a new paradigm for this homologous pairing, in essence, that recombinases such as the *E. coli* recA protein can hybridize a single strand of any sequence and an intact duplex. That is, the three strands form a novel DNA triplex (R-form DNA) in which the third strand may include both purines and pyrimidines. Thermal denaturation of chemically substituted DNAs and chemical footprinting of R-form DNA confirm that the third strand in R-form DNA is in the major groove of the duplex. We have also been able to isolate synaptic complexes consisting of all the three strands and recA. These structures have been studied in detail and will continue to be the basis of additional structural investigations. The kinetics of formation of these complexes are being used as a model for the homology search process in the obligatory recombination events during meiosis. The synaptic complexes have also been used to develop a method for the selective cleavage of human DNA (RecA-Assisted Restriction Endonuclease (RARE) cleavage); this method is now being applied to map and clone large fragments of DNA close to the Huntington Disease and Multiple Endocrine Neoplasia 1 genes. In addition, we have been successful in cloning the gene for a thermostable recA homologue. This protein should be very useful for mechanistic studies and for several important applications in biotechnology. Finally, we have cloned from yeast and higher eukaryotes several sequence homologues to recA. We are attempting to establish functional homology between the proteins encoded by these genes and the *E. coli* recA protein.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 52011-06 GBB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Toxins and DNA Repair in *Xenopus* Oocytes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Eric Ackerman Senior Staff Fellow GBB, NIDDK

Others: Shailendra K. Saxena Visiting Associate GBB, NIDDK
Timothy M. Jenkins Visiting Fellow GBB, NIDDK
Joshua D. Levin IRTA GBB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.25

PROFESSIONAL:

4.0

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

I. We previously demonstrated that the toxins α -sarcin, ricin, Shiga toxin and Shiga-like toxin all inactivate protein synthesis in cells by attacking a highly conserved region near the 3'-end of 28S ribosomal RNA and that microinjection of modified deoxyoligonucleotides, ribonucleotides and ribozymes complementary to the same region of 28S RNA also abolish protein synthesis. Surprisingly, a variety of nucleases are as effective as these toxins at abolishing protein synthesis. While most nucleases hydrolyze all cellular RNA, one of these nucleases selectively hydrolyzes tRNA, but not ribosomal or mRNAs, when injected into *Xenopus* oocytes.

II. During early development *Xenopus* replicates its DNA nearly as fast as *E. coli* in log phase. We demonstrated that oocytes are an excellent source of DNA repair activity. Pyrimidine dimer repair was shown by microinjecting UV-irradiated plasmid DNA into oocytes or adding damaged DNA to an extract derived from oocytes. We have also studied DNA repair for alkylated and chemically modified DNA as well as DNA replication with our repair extract.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 52012-08 GBB
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structure-Function Relationships of Lysosomal Enzymes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Richard L. Proia	Research Biologist GBB, NIDDK
Others:	Debra Boles	IRTA GBB, NIDDK
	Olivia Johnson	Summer IRTA GBB, NIDDK
	Francine Norflus	Biologist GBB, NIDDK
	Mark Pennybacker	IRTA GBB, NIDDK
	Shoji Yamanaka	Visiting Fellow GBB, NIDDK
COOPERATING UNITS (if any) Ruth Navon, Saphir Medical Center, Kfar-Sava, Israel		
LAB/BRANCH Genetics and Biochemistry Branch		
SECTION Biochemical Genetics Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.25	4.0	0.25
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unretouched type. Do not exceed the space provided.) <p>I. The major mutation among Ashkenazi Jewish Tay-Sachs carriers and patients is a 4 base pair(bp) insertion in exon 11 of the 14 exon HEXA gene resulting in a premature stop codon. HEXA alleles carrying the 4 bp insertion express mRNA that is rapidly degraded by an unexplained mechanism. We have developed a system to investigate the effect of the 4 bp on HEXA mRNA expression. A 8 kb "mini-HEXA gene" was constructed by fusing the coding region specified by exons 1 to 7 as a single block to a genomic segment containing the remaining exons and introns. This construct and derivatives were expressed in stably transfected L-cells and the HEXA mRNA levels were assessed by Northern analysis. We found that cells transfected with the normal construct contained readily detectable and properly spliced HEXA mRNA. Cells transfected with a mini-gene containing the 4 bp mutation, however, yielded undetectable HEXA mRNA. The mRNA level was restored with a mini-gene containing the 4 bp mutation when 1 bp was deleted upstream to correct the reading frame.</p> <p>II. Lysosomal enzyme genes exhibit an expression pattern in keeping with their role as house keeping enzymes. However, in particular tissues and under certain circumstances the expression of lysosomal enzymes is enhanced. In order to gain insight into the regulation of the lysosomal enzyme genes we have characterized the promotor region of the HEXB gene. We found that both the mouse and human HEXB genes contain a homologous 50 bp region with functional promotor activity within 40 bp of the respective RNA start sites. The region contains several binding sites for transcription factors.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 52014-05 GBB

PERIOD COVERED

October 1, 1991 to October 18, 1991

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

CD4 Receptor Structure/Function Project

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. Daniel Camerini-Otero Chief GBB, NIDDK

Others: Richard L. Proia Research Associate GBB, NIDDK
Cynthia Tift Clinical Associate GBB, NIDDK

COOPERATING UNITS (If any)

LAB/BRANCH Genetics and Biochemistry Branch

SECTION Molecular Genetics Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Project Z01 DK 52014-05 GBB is inactive.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 201 DK 52015-03 GBB												
PERIOD COVERED October 1, 1991 to September 30, 1992														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Studies of Protein-DNA Interactions														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">Peggy Hsieh</td> <td style="width: 33%;">Expert</td> <td style="width: 33%;">GBB, NIDDK</td> </tr> <tr> <td>Others:</td> <td>Tammy C. Tobin</td> <td>IRTA</td> <td>GBB, NIDDK</td> </tr> <tr> <td></td> <td>Igor G. Panyutin</td> <td>Visiting Associate</td> <td>GBB, NIDDK</td> </tr> </table>			PI:	Peggy Hsieh	Expert	GBB, NIDDK	Others:	Tammy C. Tobin	IRTA	GBB, NIDDK		Igor G. Panyutin	Visiting Associate	GBB, NIDDK
PI:	Peggy Hsieh	Expert	GBB, NIDDK											
Others:	Tammy C. Tobin	IRTA	GBB, NIDDK											
	Igor G. Panyutin	Visiting Associate	GBB, NIDDK											
COOPERATING UNITS (if any)														
LAB/BRANCH Genetics and Biochemistry Branch														
SECTION Molecular Genetics Section														
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892														
TOTAL MAN-YEARS: 3.1	PROFESSIONAL: 3.0	OTHER: 0.1												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews														
SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.) <p>DNA branch migration is an important step in both homologous recombination and site-specific recombination. Migration of the Holliday junction generated by homologous recombination can increase or decrease the amount of genetic information that is exchanged between homologues. Although there has been recent progress in determining the structure of Holliday junctions, less is known about the dynamics of branch migration particularly as it relates to the formation of heteroduplex DNA during recombination. As a first step in studying this problem, we have developed a model four-stranded system to systematically examine the effect of mispaired or unpaired bases on nonenzymatic branch migration.</p> <p>Using a series of short duplexes 40-60 bp long, we observe that the reaction consists of several distinct steps, annealing of the two duplexes, initiation of a Holliday junction and branch migration. Our results demonstrate that a single mismatched base pair impedes branch migration. Our results also indicate that the block on branch migration imposed by mismatches is more pronounced in the presence of Mg²⁺. This probably reflects the difficulty in accommodating non-Watson-Crick base pairs in the highly constrained conformation imposed on four-way DNA junctions in the presence of metal ions.</p> <p>We are also attempting to obtain a chicken cDNA clone for a type I DNA ligase. We wish to exploit the fact that chicken B cells carry out homologous recombination at unusually high frequencies to create a ligase-deficient chicken B cell line by gene targeting. In this way, we can assess the physiological function of type I ligases in these cells.</p>														

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52016-01 GBB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thyroid Hormone Interactions with Cells and Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

J. Robbins	Chief, Endocrinology Section,	GBB, NIDDK
Luigi Bartalena	Visiting Scientist	GBB, NIDDK
Marcia Phyllaieir	Biologist	GBB, NIDDK

COOPERATING UNITS (if any)

Salvatore Benvenaga (University of Messina, Italy)
Daniel Rader (NHLBI)
Mark Lakshmanan, Case Western Reserve University

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Thyroxine-binding globulin (TBG) and cortisol binding globulin (CBG) are members of the serine protease family (serpins) which include the acute phase proteins, alpha 1-antitrypsin and anti 1-antichymotrypsin. Synthesis of the latter two proteins in hepatocytes is increased by the cytokine IL-6, but synthesis of TBG and CBG are decreased. The transcription rate of the TBG gene is decreased by IL-6, while that of the CBG gene is unaltered. The decrease in CBG synthesis in the acute phase reaction, resulting in a local increase in free cortisol levels, may play a role in combating inflammation.

Continuation of work on thyroxine binding to apolipoproteins of HDL by photoaffinity labeling has now demonstrated binding not only to apoA-I but also to the minor apolipoproteins, including apoA-II, apoA-IV, apoC-I, II and III, and apoE. Thus, T₄ binding may be a general property of apolipoproteins and may have a role in the intracellular targeting of thyroid hormone.

The affinity labeling reagents, N-bromoacetyl thyroxine and N-bromoacetyl triiodothyronine have been synthesized and purified by new methods which lead to the preparation of more pure and better characterized products than were previously available. These reagents are widely used for affinity labeling of thyroid hormone binding proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 52017-01 GBB			
PERIOD COVERED October 1, 1991 to September 30, 1992					
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Studies of Thyroid Diseases					
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;"> PI: Jacob Robbins </td> <td style="width: 33%; vertical-align: top;"> Chief, Endocrinology Section </td> <td style="width: 33%; vertical-align: top;"> GBB, NIDDK </td> </tr> </table>			PI: Jacob Robbins	Chief, Endocrinology Section	GBB, NIDDK
PI: Jacob Robbins	Chief, Endocrinology Section	GBB, NIDDK			
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;"> Others: Taisheng Lee Desiree Pineda M. Phyllaier </td> <td style="width: 33%; vertical-align: top;"> Clinical Associate Clinical Associate Biologist </td> <td style="width: 33%; vertical-align: top;"> GBB, NIDDK GBB, NIDDK GBB, NIDDK </td> </tr> </table>			Others: Taisheng Lee Desiree Pineda M. Phyllaier	Clinical Associate Clinical Associate Biologist	GBB, NIDDK GBB, NIDDK GBB, NIDDK
Others: Taisheng Lee Desiree Pineda M. Phyllaier	Clinical Associate Clinical Associate Biologist	GBB, NIDDK GBB, NIDDK GBB, NIDDK			
COOPERATING UNITS (if any) J. Norton, Surgery Branch, NCI; J. Reynolds, Nuclear Medicine, CC; M. Merino, Laboratory of Pathology, NCI; C. Meyers, NCI					
<table style="width: 100%; border: none;"> <tr> <td style="width: 20%;">LAB/BRANCH</td> <td>Genetics and Biochemistry Branch</td> </tr> </table>			LAB/BRANCH	Genetics and Biochemistry Branch	
LAB/BRANCH	Genetics and Biochemistry Branch				
<table style="width: 100%; border: none;"> <tr> <td style="width: 20%;">SECTION</td> <td>Endocrinology Section</td> </tr> </table>			SECTION	Endocrinology Section	
SECTION	Endocrinology Section				
<table style="width: 100%; border: none;"> <tr> <td style="width: 20%;">INSTITUTE AND LOCATION</td> <td>NIDDK, NIH, Bethesda, Maryland 20892</td> </tr> </table>			INSTITUTE AND LOCATION	NIDDK, NIH, Bethesda, Maryland 20892	
INSTITUTE AND LOCATION	NIDDK, NIH, Bethesda, Maryland 20892				
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">TOTAL MAN-YEARS:</td> <td style="width: 33%;">PROFESSIONAL:</td> <td style="width: 33%;">OTHER:</td> </tr> </table>			TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:			
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;"> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </td> <td style="width: 33%; vertical-align: top;"> <input checked="" type="checkbox"/> (b) Human tissues </td> <td style="width: 33%; vertical-align: top;"> <input type="checkbox"/> (c) Neither </td> </tr> </table>			<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither			
SUMMARY OF WORK (Use standard unretouched type. Do not exceed the space provided.) <p>Work has continued on the use of low dose doxorubicin as a radiation enhancer to improve the effectiveness of I-131 in the treatment of high-risk thyroid cancer. Twenty patients have entered the protocol, 7 of whom were randomized to receive the combination therapy. A total of 19 I-131 treatments combined with adriamycin have been administered with no apparent additional toxicity.</p> <p>A second group of thyroid cancer patients has taken part in a study to test the effectiveness of recombinant human TSH to stimulate uptake in the thyroid remnant following initial thyroidectomy. This Phase I and II study to test efficacy and toxicity is part of a multi-institutional effort in collaboration with Bruce Weintraub and the Genzyme Corporation. The initial results are under evaluation.</p> <p>A third group of thyroid cancer patients are under study to evaluate the use of the serum thyroglobulin (TG) level as a guide to I-131 therapy in patients in whom a diagnostic whole body I-131 scan is negative. Fourteen patients have received up to three treatments with 150 to 300 mCi I-131 and the post therapy scan was positive in 13 patients (in 21 of 28 treatments). Serum TG decreased in 82% of patients, and reverted to ≤ 5 ug/ml in 29% of patients after one or two treatments. The post therapy whole body scan became negative in 50% of patients after two or three treatments.</p>					

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52018-01 GBB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of Thyroid Hormone on Synthesis of Myelin Basic Protein

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI	V. Nikodem	Senior Investigator
	A. Farsetti	Visiting Fellow
	F. Bogazzi	Visiting Fellow
	D. Pineda	Clinical Associate
	B. Dozin-Quarto	Visiting Scientist

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Mechanisms of Gene Regulation

INSTITUTE AND LOCATION

TOTAL STAFF YEARS:

3

PROFESSIONAL:

3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

1. In this study we characterized further the myelin basic protein thyroid hormone response element by functional and binding analyses. The results revealed that this element is composed of two regions each of them containing CC or GG nucleotide pairs which are essential for this response element function, since mutation in either of them abolished thyroid hormone responsiveness. Computer assisted comparison of several thyroid hormone response elements revealed, that these pairs are always separated by eight nucleotides. Furthermore, these elements could be arranged as direct repeats or inverted palindromes. We are constructing mutants of both elements to explore a potential role of corresponding nucleotides in these two structurally different elements.

2. Several studies have indicated that thyroid hormone receptors can form various oligomeric complexes with nuclear proteins. Liver and brain nuclear extracts from embryo, 3 and 20 days after birth and adult rats were used in gel shift mobility assays either alone or mixed with thyroid hormone receptors prior to the addition of labeled malic enzyme and myelin basic protein thyroid hormone response elements. Using either element, three complexes were detected in the presence of the added receptor and any liver extract. These complexes were also detected by the labeled hormone. Only the receptor homodimer was seen with the malic enzyme element in the presence of any brain extract. However, a complex, in addition to the receptor homodimer, was detected when only brain nuclear extract from embryo and 3 days old brain was used in the presence of myelin basic protein element. Thus, the "so called" thyroid hormone non-responsiveness of the adult brain might be explained by the absence of a nuclear protein.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 52019-01 GBB
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Biology of Thyroid Hormone Receptor		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: V. Nikodem Senior Investigator GBB, NIDDK		
Others: E. Jannini Guest Worker GBB, NIDDK P. Hallenbeck Staff Fellow GBB, NIDDK R. Lippoldt Chemist GBB, NIDDK M. Phyllaier Technician GBB, NIDDK		
COOPERATING UNITS (if any)		
LAB/BRANCH Genetics and Biochemistry Branch		
SECTION Mechanisms of Gene Regulation		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 3.5	PROFESSIONAL: 2	OTHER: 1.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>1. The thyroid hormone receptor α gene is alternatively spliced to generate the α receptor and a variant, which does not bind thyroid hormones. An additional α like receptor, Rev-erbAα, is transcribed on the opposite strand overlapping the unique variant sequences. The overlapping region of these two transcripts can form a sense-antisense hybrid and thus modulate a level of variant with concomitant increase in production of the α receptor. Our results showed that transcription of these two genes from the erbA α genomic locus is independent of each other, since the level of variant is very high at any developmental stage, the level of receptor is very low with transient increase 15 days after birth and the Rev-erbAα is high only in the adult tissues.</p> <p>2. We are interested in the precise mechanism by which thyroid hormone receptor and other ligand activated-nuclear receptors induce or repress transcription of genes in response to a ligand. We and others have demonstrated that at least part of this mechanism involved the ability of the thyroid hormone receptor to heterodimerize, with another hormone receptor. Several questions arise. What is a role of the ligand, one or both, in a heterodimer binding to different response elements? To answer these questions and study various kinetic parameters we have begun to purify thyroid hormone receptor. We have made plasmids expressing a fusion protein, in which the N-terminus of the receptor is fused with polyhistidine. Baculovirus and E. coli expression systems have been employed. A nickle column has been used to purify overexpressed protein.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 52020-01 GBB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Regulation of Anteroposterior Patterning in Early Frog Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
PI: S. Sato Senior Staff Fellow GBB, NIDDK

Others: V. Agarwal Visiting Associate GBB, NIDDK
S. Witta Visiting Fellow GBB, NIDDK

COOPERATING UNITS (if any)
Dr. William Hayes, LDN, NICHD

LAB/BRANCH Genetics and Biochemistry Branch

SECTION Mechanisms of Gene Regulation

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.2	PROFESSIONAL: 0.0	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects
☐ (a1) Minors
☐ (a2) Interviews
☐ (b) Human tissues
☒ (c) Neither

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The POU domain gene family has been shown to be important in tissue specific gene regulation during development. It has been proposed that POU class III transcription factors act in a combinatorial fashion to establish various neuronal phenotypes in the brain. We are investigating the role of two of these POU class III factors, XLPOU 1 and XLPOU 2, in early neural development. In situ hybridization analysis of *Xenopus* embryos has demonstrated that in the neural plate, XLPOU 1 gene expression is restricted to two symmetrical patches corresponding to the future midbrain and hindbrain. In tailbud stage embryos, additional XLPOU 1 gene expression is observed in the forebrain and eyes. XLPOU 2 gene expression is observed in the ventral forebrain, midbrain, and hindbrain. The restricted pattern of gene expression observed with these POU domain genes is unique because these genes are expressed in brain regions more anterior to most of the various homeobox 1 and XLPOU 2 gene expression is greatly affected by retinoic acid (RA) treatment of embryos. The anterior boundary of both of these genes is shifted in the brains of the RA-treated embryos. Furthermore, with moderate doses of RA, XLPOU 1 gene expression is greatly enhanced in the midbrain and eye. XLPOU 1 and XLPOU 2 should prove to be useful markers in studying how the anterior part of the brain is established.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 52021-01 GBB
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mapping of Triiodothyronine Responsive Genes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> PI: V. Nikodem Senior Investigator GBB, NIDDK </div>		
<div style="display: flex; justify-content: space-between;"> <div> Others: J. Lazar B. Desvergne </div> <div> Visiting Fellow Visiting Associate </div> <div> GBB, NIDDK GBB, NIDDK </div> </div>		
COOPERATING UNITS (if any)		
LAB/BRANCH Genetics and Biochemistry Branch		
SECTION Mechanisms of Gene Regulation		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 2	PROFESSIONAL: 2	OTHER: 0
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews </div> <div> <input type="checkbox"/> (b) Human tissues </div> <div> <input checked="" type="checkbox"/> (c) Neither </div> </div>		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) <p>1. Regulated expression of thyroid hormone receptor genes provides an additional means of controlling the action of thyroid hormone. We cloned and sequenced a 5' flanking region of the rat thyroid hormone receptor α gene, which revealed no presence of TATA and CAAT boxes. Using primer extension assays multiple extension products were observed. A minimal region necessary for promoter activity resides within 193 nucleotides upstream from the major start of transcription. Further analyses showed that a strong positive element is in the splicing junction of the untranslated exon/intron. Only in the presence of the hormone and the receptor the promoter activity was inhibited by 50 - 80%.</p> <p>2. Identification of malic enzyme and myelin basic protein thyroid hormone response elements in different promoters allowed us to study thyroid hormone dependent activation of these promoters by the receptor α and β. We have demonstrated that the more efficient thyroid hormone responsiveness of the myelin basic promoter elicited by the receptor β depends on both the thyroid hormone response element of the myelin basic protein promoter and TATA box sequences. In the case of the malic enzyme promoter, the more efficient thyroid hormone dependent activation by the α receptor requires the thyroid hormone response element of the malic enzyme gene and an element within 122 nucleotides upstream from the start of transcription of this gene.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52022-09 GBB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Specific Rat Liver mRNAs by Thyroid Hormone

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. (Name, title, laboratory, and institute affiliation))

PI: V. Nikodem

Others: R. Lippoldt
J.E. Rall

CEB, NIDDK
CEB, NIDDK

COOPERATING UNITS (If any)

LAB/BRANCH

Genetics and Biochemistry branch

SECTION

Mechanisms of Gene Regulation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

0

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

This project is inactive.

ANNUAL REPORT OF THE DIGESTIVE DISEASES BRANCH, NIDDK

Summary of Branch Activities

All four sections of the Digestive Diseases Branch are engaged in investigations of basic biologic processes leading to alteration in the function of gastrointestinal tissues and are attempting to apply this information to understand the pathophysiology of various disorders involving the liver, pancreas and gastrointestinal tract. All four sections are also involved in attempts to improve therapy of clinical disorders such as neoplasms associated with overproduction of gastrointestinal hormones, hepatitis and fulminant hepatic failure. Below the important results found in the various sections are briefly summarized with more detailed descriptions in the individual section reports.

I. Section on Gastroenterology

A. *Identification and characterization of receptors for GI peptides*

1. Identification of receptors for GI peptides. Recent studies by us and others provided evidence that the mammalian bombesin (Bn) related peptides, gastrin-releasing peptide (GRP) and neuromedin B (NMB), alter cell function by interacting with two distinct subtypes of receptors. We have now developed agonists and selective antagonists that distinguish these 2 subtypes. During this year in collaboration with T. Moran (Johns Hopkins University) and T.W. Mody (George Washington University) both NMB and GRP receptors were found in the CNS. Furthermore the NMB receptors were the predominant subtype explaining why in previous studies various selective GRP receptor antagonists did not alter Bn stimulated functions *in vivo* in the CNS. The existence of 2 distinct Bn receptor subtypes was proven during the year with the cloning of the NMB receptor by Jim Battey (NCI). In collaborative experiments with J. Battey the cloned expressed receptor was shown to have the characteristic pharmacology of a NMB receptor.

Recent studies suggest that GI peptides may have important effects on altering growth of various neoplasms. For example, recent studies report colon cancers possess gastrin receptors and that gastrin affects their growth. Because hypergastrinemia is common in humans and some studies show increased gastrin levels in patients with colonic adenomas/carcinomas we examined 10 different human colonic cell lines for the occurrence of gastrin receptors as well as to 11 other GI hormones and neurotransmitters. Receptors for muscarinic cholinergic agents or VIP occurred in 60%, bombesin or gastrin in 30%, β -adrenergic agents and GRP in 20% and somatostatin, opiates, NMB and substance P in 10%. Pharmacological analysis demonstrated the various receptors resembled those on normal gastrointestinal tissues. Furthermore, the presence or absence of receptors detected by binding studies correlated closely with the ability of selective receptor agonists to alter cell function (changes in cytosolic calcium or phosphoinositides). These studies are currently being extended to determine whether receptor occupation by agonists affects tumor growth.

A number of different observations suggest that the GI hormone, CCK, can alter growth of both normal and neoplastic pancreatic tissue and that CCK can increase the development of experimentally induced pancreatic cancer. In a collaborative study with R. Bell (University of Cincinnati; VA Medical Center) it was shown that neoplastic tissues have greater than a two-fold increase in CCK receptors and this change is also seen on pre-neoplastic adenomatous nodules. The increase is primarily due to an increase in the number of high affinity CCK receptors. These studies are currently being extended by determining the receptor subtype increased in numbers and which subtypes are responsible for tumor growth promotion.

2. Characterization of receptors for gastrointestinal hormones and their possible physiological importance by developing specific receptor antagonists. In previous studies we have developed 4 different classes of Bn receptor antagonists and in recent studies demonstrated some are highly selective for the GRP receptor subtype. From these studies as well as other structure function studies it was proposed that similar to LHRH and somatostatin, the COOH terminus of Bn on GRP may possess a β -turn between Val¹⁰ and Leu¹⁴. Molecular modeling studies support this conformation and suggest that a hydrophobic pocket projects above the plane of the molecule and is crucial for determining receptor affinity. Structure-function studies demonstrated alterations in the hydrophobic

residues in this pocket had a profound effect on receptor affinity. Modeling studies suggest because of the β -bend the NH_2 and COOH termini are in close proximity in this proposed foiled configuration and this was further supported by demonstrating that cyclization of Bn joined either by a disulfide band or a head-to-tail peptide linkage, functioned as Bn receptor agonists. Insertion of a D-Ala¹¹ into the cyclized peptide, a group known to stabilize β -turns, increased the affinity, further supporting this proposed configuration as the active one.

In our recent studies we demonstrated that des Met¹⁴ Bn analogues such as [D-Phe⁹] Bn(6-13) methyl ester were extremely potent Bn receptor antagonists both *in vitro* and *in vivo*. Because it is important to have a long-acting antagonist for may *in vivo* studies, we altered a number of residues of this antagonist to attempt to increase its potency *in vivo*. Insertion of a [D-Ala¹¹] increased *in vivo* duration by 50%, whereas insertion of the lipophilic residue [D-penta-fluoro-D-Phe⁹] at the NH_2 terminus increased *in vivo* duration of action by 15-fold with *in vivo* receptor blockade lasting > 4.5 hours. This analogue thus appears to be a good candidate for clinical studies where prolonged blockade of endogenous bombesin-like peptide is desirable.

B. Cellular basis of action of gastrointestinal peptides

1. Cellular basis of action of bombesin (Bn) related peptides at NMB receptors: We have recently discovered a specific receptor for the mammalian Bn-related peptide, NMB in rat esophageal muscularis mucosa which has been recently cloned from this tissue by J. Battey (NCI, NIH). Northern analysis suggested this receptor also existed in the rat glioblastoma tumor cell line, C-6. In this study we demonstrated that the NMB receptor in C-6 cells was pharmacologically indistinguishable from that on the rat esophageal muscularis mucosa or the NMB receptor from rat esophageal muscularis mucosa transfected into Balb 3T3 cells. Previous studies showed that agonist occupation of the receptor for the Bn related peptide GRP caused both phospholipase C activation and increases in cAMP. In C-6 cells, NMB activated phospholipase C increasing cytosolic calcium and various phosphoinositides including IP_1 , IP_2 and IP_3 . Stoichiometric studies demonstrated that these were spare receptors on these cells in that submaximal receptor occupation caused maximal changes in $[\text{Ca}^{2+}]$ or IP_3 . In contrast to GRP receptors, NMB caused no alteration in cAMP at NMB receptors.

Bn-related peptides are known to have potent growth effects and to function as an autocrine growth factors in a number of tumors including human small cell lung cancer cells (SCLC cells). This has been thought to be mediated by GRP receptors. In collaboration with T.W. Moody (Biochemistry Dept., George Washington University), it was demonstrated some SCLC lines also possess NMB receptors and that occupation of these receptors by NMB activates phospholipase C, increases cytosolic calcium and also results in growth.

2. Cellular basis of action of gastrin and cholecystokinin (CCK) to stimulate pepsinogen release from chief cells. Our previous studies have demonstrated the structurally related peptides CCK and gastrin can stimulate pepsinogen release from dispersed chief cells, however it is unclear whether 1 or 2 receptors mediate these changes or what the intracellular mediators are. In this study both CCK and gastrin were found to increase pepsinogen release, phosphoinositides and alter cytosolic calcium ($[\text{Ca}^{2+}]$). However, they differed in efficacy, potency and the configuration of their dose-response for altering these cellular processes. Using the highly selective CCK_A agonist, A-71378 and the antagonist L-364,718 it was found that gastrin and CCK were altering cellular function by interacting with two distinct receptors, a CCK_A and a CCK_B /gastrin receptor. Both receptors were coupled to phospholipase C and caused changes in inositol phosphates, cytosolic calcium and pepsinogen release, however the intracellular amplification differed between the two receptor subtypes. Activation by CCK-related peptides at the CCK_A receptor subtype accounted for 85 - 90% of the maximal changes in cellular function and activation of the CCK_B /gastrin receptor accounted for 10 - 20% of maximal changes.

3. Role of calcium in secretagogue-induced secretion from pancreatic acinar cells. Controversy exists about the role of extracellular and intracellular calcium in enzyme secretion in pancreatic acini. In this study, thapsigargin (TG), which inhibits microsomal calcium ATPase thus

blocking calcium uptake into microsomal stores, was used. In collaboration with R.J. Turner (NIH, NIDR), using single cell microspectrofluorimetry of fura-2 loaded cells, TG alone caused a dose-related increase in $[Ca^{2+}]_i$ by increasing Ca influx and mobilization from an $IP_3(1,4,5)$ sensitive pool. TG caused an increase in basal amylase release, inhibited release by Ca mobilizing secretagogues and potentiated release by agents that increase cAMP or by TPA. These results demonstrated that amylase secretion by secretagogues that increase $IP_3(1,4,5)$ does not depend on increased free cytoplasmic calcium *per se*. In contrast, TG-induced potentiation of stimulation by secretagogues that increase cellular cAMP appears to result from increased free cytoplasmic calcium *per se*.

4. Action of VIP-secretin-PACAP related peptides on pepsinogen release from chief cells. In previous studies we have demonstrated VIP and secretin, which are structurally related, can stimulate pepsinogen release from chief cells. PACAP (pituitary adenylate cyclase activating peptide) is a 38 amino acid with structural similarity to these peptides and in other tissues, specific high affinity receptors are present for this peptide which also interacts with VIP and secretin. PACAP38, PACAP27, VIP and secretin all stimulated pepsinogen release, however, they differed in potency and in the configuration of their dose-response curves. With PACAP 38, PACAP27 and VIP but not secretin the dose-inhibition curves were biphasic. Each peptide inhibited ^{125}I -PACAP27, ^{125}I -VIP and ^{125}I -secretin binding and each stimulated cyclic AMP generation. Comparison of binding curves, ability to increase cAMP and pepsinogen release demonstrated that these cells possess 2 classes of receptors mediating the action of these peptides. PACAP and VIP interact with high affinity with a common receptor to stimulate pepsinogen release and at low affinity with a secretin receptor to stimulate pepsinogen release. With PACAP occupation of PACAP-VIP receptors causes 40% of maximal release and at higher concentrations occupation of secretin receptors causes the remaining 60% of maximal secretion.

C. Receptors on gastric smooth muscle cells

1. Role of cAMP in mediating gastrointestinal smooth muscle relaxation by β -adrenergic agents or neuropeptides. Numerous studies suggest that gastrointestinal smooth muscle relaxation caused by β -adrenergic agents or neuropeptides occurs as a result of increases in cAMP. However, the evidence is in direct and does not demonstrate that the increase in cAMP is essential for mediating relaxation. To more clearly define the role of cAMP in receptor mediated GI smooth muscle relaxation we used the protein kinase A competitive antagonist [(R)3',5', cyclic phosphothioate] (Rp-cAMPS) and its isomer Sp-cAMPS which functions as an agonist. Rp-cAMPS inhibited by 80% the ability of Sp-cAMPS, VIP, isoproterenol, CGRP and glucagon to stimulate relaxation, demonstrating each of these agents is causing relaxation primarily through activation of PKA. In contrast, relaxation by ATP or sodium nitroprusside was not effected. These data demonstrate that activation of PKA is essential for mediating at least 80% of the gastric smooth muscle relaxation produced by β -adrenergic agents or neuropeptides.

2. Cellular basis of action of somatostatin on gastric smooth muscle. Somatostatin is known to alter gastric emptying and GI motility. To investigate its cellular basis of action we investigated the ability of the natural occurring somatostatins, somatostatin-14 (SS-14), somatostatin-28 (SS-28) and a synthetic analogue, D-Phe,Cys,Tyr-D-Trp-Lys-Thr-Cys-Nal-NH₂ (Cyclo SS-8) to alter activity of dispersed gastric smooth muscle cells. The somatostatins did not cause contraction or relaxation when present alone, but inhibited the relaxant effects of VIP. SS-28 and cyclo SS-8 caused half-maximal effects at 1 nM, whereas SS-14 had no effect at concentrations up to 1 μ M. Addition of the protease inhibitors phophoramidon and amastatin increased the potency of SS-14 > 1,000 fold. HPLC analysis demonstrated SS-14 was being rapidly degraded and the protease inhibitors blocked this degradation. Cyclo SS-8 inhibited the relaxant effects of VIP, isoproterenol, glucagon and dibutyryl cAMP and its inhibitory action was partially blocked by pertussis toxin. Cyclo SS-8 significantly inhibited VIP stimulated generation of cAMP. These results indicate that gastric smooth muscle rapidly degrade SS-14 and suggest that smooth muscle proteases could have a major effect on determining the duration of action of SS-14. Furthermore, gastric muscle cells possess somatostatin receptors, occupation of which inhibits relaxation. This inhibitory effect is mediated

both through a guanine nucleotide regulatory protein G_i to inhibit adenylate cyclase and at sites distal to the generation of cAMP.

3. Characterization of opioid receptors on gastric smooth muscle cells. A number of studies have suggested gastrointestinal smooth muscle cells as well as gastrointestinal neural elements have specific opioid receptors. To investigate the opioid receptors present on GI muscle and their abilities to interact with opioid peptides directly, we examined the ability of various selective opioid ligands and selective agonists and antagonists to interact with dispersed gastric muscle cells. The kappa selective agonist U-50488H, the μ agonist DAGO, and the delta agonist DPDPE all caused muscle contraction. Experiments with selective antagonists demonstrated that κ and μ receptors were present but that stimulation by the delta agonist DPDPE was due to κ and μ receptor activation. Binding studies with μ and κ ligands confirmed the presence of these receptors and no binding was seen with a δ ligand. These data demonstrated that gastric smooth muscle cells possess κ and μ receptors, occupation of which causes muscle contraction, but no δ receptors were detected.

II. Section on cell biology

Molecular characterization of receptors for GI peptides

A. Cloning of the cholecystikinin (CCK) type A receptor (CCK_A receptor). At least 2 classes of receptors mediate the effects of CCK and gastrin which structurally resembles CCK. The CCK_A receptor is thought to have numerous functions both in the CNS and peripheral tissues including effects on satiety, pancreatic secretion, gallbladder contraction and potentiation of insulin release. A number of groups have attempted either structural determination of receptor sequence from isolated receptors or molecular biology studies but because of the small receptor number they had been unsuccessful.

In this study we developed a method to purify the CCK_A receptor protein to homogeneity (14,600-fold purification) from rat pancreas using cationic exchange resin, wheat germ agglutinin and blue Sepharose chromatography after solubilization with 1% digitoxin. The receptor was blocked at the NH₂ terminus to Edman degradation, however cyanogen bromide cleavage or Lys C digestion with o-phthalaldehyde blocking allowed sequencing of 5 peptides. Degenerate oligonucleotide primers were synthesized and using PCR with single stranded cDNA reverse transcribed from rat pancreatic mRNA a partial sequence (527 BP) was obtained. This sequence was used to screen an oligo-(dT)-primed cDNA library from rat pancreas and 6 clones were identified, however all lacked the 3' end of the coding sequence. The remaining sequence was obtained using various PCR methods inducing a RACE protocol and anchored PCR. A 1506 BP sequence was identified which encoded a 444 amino acid peptide which contained the 5 peptide sequences identified from CNBr and Lys C-digestion of the purified receptor. A hydrophathy plot demonstrated 7 putative transmembrane domains characteristic of G-protein coupled receptors. The protein was novel having only 30% amino acid homology with the closest similar receptors (neuromedin K, substance P, GRP). High-stringency Northern blot analysis demonstrated a 2.7 Kb transcript in rat pancreas and AR42J cells with no hybridization in tissues thought not to possess CCK_A receptors. In vitro transcripts of the cDNA functionally expressed in *Xenopus* oocytes demonstrated a response to CCK (and not to GRP or acetylcholine) which was blocked by the CCK_A specific antagonist L-374,718. Transient expression in COS-7 cells transfected with the CCK_A receptor cDNA demonstrated the expected binding affinities of high affinity for CCK-8 or the CCK_A antagonist L-364,718 and low affinity for gastrin and the CCK_B antagonist, L-365,260.

B. Cloning of the CCK_B/gastrin receptor. The CCK_B/gastrin receptor is the predominant CCK receptor in the CNS modulating anxiety, neuroleptic activity, opiate induced analgesia and arousal; whereas in peripheral tissues CCK_B receptors modulate growth effects of CCK/gastrin on pancreas, stomach and various tumor cell lines; motility and effects on the immune system.

In this study we used a random primed probe corresponding to the coding sequence of the CCK_A receptor under conditions of low stringency to screen a cDNA library from AR42J cells, a

pancreatic tumor cell line known to have high concentrations of CCK₈/gastrin receptors. A novel 2,243 bp clone was isolated which encoded a 452-amino acid protein that had 48% identity to the CCK_A receptor. Screening of a rat brain cDNA library demonstrated clones identical to the clones from AR42J cells. The CCK₈/gastrin receptor had 4 potential N-linked glycosylation sites, one potential site for PKC phosphorylation in the 1st intracellular loop and 2 potential sites for PKA phosphorylation, one in the 2nd intracellular loop and the other in the cytoplasmic tail. Hydropathy plot suggested 7 transmembrane domains characteristic of G-protein coupled receptors. Northern blot analysis revealed 2.7 Kb band in rat brain and AR425 cells with no hybridization in tissues not known to contain CCK₈/gastrin receptors. Transient transfection in COS-7 cells demonstrated the expected pharmacology with approximately equal high affinity for the agonist gastrin and CCK-8 and a 30 fold higher affinity for the CCK₈/gastrin receptor antagonist L-365,260 than the CCK_A antagonist L-364,718.

III. Section of clinical investigation

Management of islet cell tumors

The clinical investigation section of the Digestive Diseases Branch is now following 200 patients with Zollinger-Ellison syndrome (ZES) caused by a neuroendocrine tumor of the pancreas or duodenum which autonomously releases gastrin which causes gastric acid hypersecretion. A smaller numbers of patients with other islet cell tumors are also being followed. All patients with ZES have two problems: gastric acid hypersecretion; and the malignant nature of the tumor itself. This section is involved in studies dealing with both areas.

Although increasingly effective gastric acid antisecretory agents such as the H⁺-K⁺ ATPase inhibitor omeprazole have been developed and shown to be extremely effective in patients with ZES a number of problems remain unresolved. These include high dose requirements which in some patients are prohibitively expensive (> \$20/day in some patients); therefore more potent agents are needed. We demonstrated that the new H⁺-K⁺ ATPase antagonist, lansoprazole, has a long duration of action (> 36 hours) and is effective and safe in 20 patients with ZES. Long-term comparative studies with omeprazole need to be done to establish whether there are important clinical differences between these two agents.

The FDA recommended daily dose of omeprazole based on our studies and studies by others is 60 mg/day. A large percentage of ZES patients require > 120 mg/day, a dosage determined by acute acid titration studies. Because the efficacy of this drug increases in the first week of treatment, we prospectively assessed the possibility that these maintenance doses were too high in 37 patients with ZES. Our results showed that 68% of patients could be successfully reduced to a dose of 20 mg QD or 20 mg BID of omeprazole. Furthermore in patients with uncomplicated disease (no MEN-1, severe esophageal reflux or previous gastric surgery) the dose could be reduced in 95% of patients. These studies show that the currently recommended omeprazole maintenance dose in patients with ZES is too high. This is an important conclusion because most patients (~70%) with ZES are currently not cured and thus need to take lifelong omeprazole. By following the protocols outlined in this study, it will be possible to reduce the expense of omeprazole in many patients with ZES taking omeprazole once daily by \$3,600 and in those taking it twice per day by \$6,000. This should also result in increased compliance because many of the patients cannot afford the high cost of these agents at existing doses.

ZES is the most common malignant islet cell tumor and increasingly with the ability to medically control gastric acid hypersecretion the malignant nature of the gastrinoma is becoming the primary determined of long-term cure. Because <30% of patients are reported cured, we have attempted to identify better methods to localize the tumor as well as remove it at surgery. In collaboration with the Clinical Center Radiology Department (Principal Collaborator, J.D. Doppman), functional localization using portal venous sampling for gastrin has been extensively studied. Our results show localization in 70% of patients, however significant morbidity (pain at sampling site) occurs in 20-30%. By using secretin provocation during the angiogram with hepatic venous sampling

for gastrin concentrations, we have been able to localize gastrinomas in 90% of patients and this method is more sensitive for localizing duodenal tumors. Because of its increased sensitivity as well as greater simplicity and less morbidity, our studies show this test should replace the portal venous sampling. Furthermore, recent alterations of this technique using small bolus injections of calcium has proved useful for identifying insulinomas.

In a collaborative study with the Radiology Department (Clinical Center, PI - J.D. Doppman) we have prospective studied the ability of magnetic resonance imaging (MRI) to localize primary and metastatic gastrinomas. Older studies by us and others showed MRI was not sufficiently sensitive to be generally useful, however recent advances in MRI scanning have greatly improved sensitivity. Our study showed for metastatic tumors in the liver the sensitivity of MRI was 83%, ultrasound 50%, CT scanning 50% and angiography 61%. The specificity for MRI was 90% and there this study shows MRI is now the imaging study of choice for assessory metastatic gastrinoma. For localizing and assessing primary tumors selective angiography remains the procedure of choice.

In collaborative studies with JA Norton (NCI, Surgery Branch) during this last year we analyzed our 10 year prospective study in patients with ZES of the ability to surgically localize and resect gastrinomas and cure patients long-term. Our results show that from 1981 - 1985 we were able to localize gastrinomas in 70% of patients and from 1986 - present in 92%. The increased localization was due to a combination of using intraoperative ultrasound, transillumination of the duodenum at surgery and duodenotomy. Immediately postoperatively 60% of patients have normal gastrins and at 5 years 30% had normal gastrins and negative provocative tests which is a marked increase in long-term cure rate compared to those reported previously. These results suggest that by the increased use of functional localization studies, imaging studies and operative localization techniques, the possibility of long-term cure in these patients can be markedly improved.

SUMMARY

Liver Diseases Section

The Liver Diseases Section is currently responsible for four principal projects.

I. Studies Relating to the Pathogenesis of Hepatic Encephalopathy and Fulminant Hepatic Failure

Ameliorations of hepatic encephalopathy (HE) (both clinical and electrophysiologic) have been induced in animals with fulminant hepatic failure (FHF) by benzodiazepine (BZ) receptor antagonists. Furthermore, Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and, in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. These findings suggest that in HE due to FHF: (i) There is increased GABAergic tone; (ii) Blocking of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) An endogenous BZ receptor agonist may contribute to HE. Increased levels of 1,4-BZs have been demonstrated in the brain of models of HE and humans with FHF. The efficacy of BZ receptor ligands in ameliorating HE in animal models does not appear to be dependent on their intrinsic activity but may be related to their affinity for BZ receptor subtypes in addition to the diazepam sensitive receptor. In vivo and in vitro studies indicate that the hepatocellular cytoprotective effects of the prostaglandin PGE₂ are mediated via activation of cAMP. (E.A. Jones, J. Vergalla, C. Yurdaydin, M.G. Swain, N.V. Bergasa, A.S. Basile, P. Skolnick, S.M. Paul, non NIH: R.B. Rothman).

II. Trials of Therapies for Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is a disease of unknown etiology characterized by slowly progressive intrahepatic cholestasis due to nonsuppurative, presumably autoimmune, destruction of small bile ducts. As some other autoimmune diseases appear to respond favorably to certain immunosuppressive agents, trials of selected immunosuppressive drugs are being undertaken in patients with PBC. Ten patients with symptomatic PBC are being treated in an open trial of low-dose (15 mg once weekly) oral methotrexate. Six patients have been followed for over 2 years. Elevated serum IgM and alkaline phosphatase levels decreased significantly and symptom scores for fatigue and pruritus fell by over 50%. Serum ALT levels initially increased but subsequently fell below baseline. Methotrexate therapy was also associated with a substantial decrease in hepatic inflammation but no obvious change in hepatic fibrosis. Twenty-five patients have been entered into a randomized trial of two doses of methotrexate (7.5 and 15 mg/week) for asymptomatic as well as symptomatic patients with PBC (E.A. Jones, J.H. Hoofnagle, N.V. Bergasa, M.G. Swain, C. Yurdaydin, R. Sallie, S.C. Chia).

III. Studies of Cellular Immune Function in Primary Biliary Cirrhosis

The role of abnormal immune mechanisms in the mediation of the hepatobiliary lesion of primary biliary cirrhosis (PBC) is being studied. CD4+, Leu-8+ T cells from patients with PBC, but not from patients with other liver diseases, have been shown to exhibit a defect in their ability to suppress immunoglobulin synthesis by B cells in vitro. Furthermore the proliferative responses of these cells from patients with PBC to mitogenic stimulation was found to be impaired. Exposure of this subpopulation of T cells from patients with PBC to phorbol ester, which activates the protein kinase C pathway, corrects their abnormal function. Thus a defect in the biochemical pathway involving protein kinase C may contribute to the immunological abnormalities exhibited by patients with PBC. In contrast to control patients with non-PBC chronic inflammatory liver

diseases, mRNA for IL-1, 2, 4, 5 and 6, and TNF- α were not detected in liver biopsies from patients with PBC. These findings suggest that immunologic injury that is not mediated by cytokines plays a major role in disease progression in PBC. (E.A. Jones, M. Shindo, N.V. Bergasa, J. Vergalla, R. Sallie, non-NIH: S.P. James.

IV. Studies of the Opiate System in Cholestatic Liver Disease

Pruritus is a common distressing complication of cholestatic liver diseases. The hypothesis that endogenous opioid agonist ligands may contribute to this symptom is suggested by the precipitation of an opiate withdrawal-like syndrome by an opiate antagonist in patients with chronic cholestatic liver disease; and the recognized ability of exogenously administered opiates to induce pruritus. To test this hypothesis the effects of infusing naloxone in pruritic patients with primary biliary cirrhosis has been assessed using a newly designed device which enables scratching activity to be continuously recorded independent of coarse body movements. In both a single blinded controlled trial and a double-blind placebo-controlled trial naloxone has been shown to reduce scratching activity substantially in such patients. Further studies of the opiate system in cholestasis in the rat revealed that bile duct resection induces a state of antinociception which can be stereoselectively reversed by naloxone, down-regulation of mu-opioid receptors in the brain and expression of preproenkephalin in the liver. These findings suggest that in cholestasis there is increased opiate tone which could contribute to the pruritus that complicates this syndrome. Long term amelioration of this form of pruritus may be possible by administering an opiate antagonist that is effective when given orally such as nalmefene. Preliminary experience with the oral administration of nalmefene for the pruritus of chronic cholestasis in an open label trial has been encouraging and a double-blind placebo-controlled nalmefene trial has been initiated. (E.A. Jones, N.V. Bergasa, J. Vergalla, C. Yurdaydin, R. Sallie, M.G. Swain, S. Sabol, non NIH: R.B. Rothman).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53001-21 DDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (20 characters or less. This must fit on one line between the borders.)

Studies of Membrane Function

PRINCIPAL INVESTIGATOR (Last name, professional acronym, below the Principal Investigator's Name, title, laboratory, and institute affiliation)

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

This project has been combined with project Z01 DK 53100 DDB.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 53004 DDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cyclic Nucleotide Mediated Functions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES):

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

This project has been combined with project 201 DK 53101 DDB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53100-04 DDB

PERIOD COVERED

October 1, 1991, to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification and characterization of receptors for GI peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R.T. Jensen	Branch Chief	DDB, NIDDK
Others:	S.A. Wank	Senior Investigator	DDB, NIDDK
	S.A. Mantey	Chemist	DDB, NIDDK
	J. Mrozinski, Jr.	Chemist	DDB, NIDDK
	P. Hildebrand	Special Volunteer	DDB, NIDDK
	T. Honda	Visiting Associate	DDB, NIDDK
	V.A. Fishbeyn	Clinical Associate	DDB, NIDDK
	M. Orbuch, R. Benya	Clinical Associates	DDB, NIDDK

COOPERATING UNITS (If any)

Tulane University, New Orleans, LA (D.H. Coy); VA Medical Center, Cincinnati, OH (R.Bell); Johns Hopkins School of Medicine, Baltimore, MD (T.H. Moran); George Washington University, Washington, DC (T.W. Moody); NCI, LBC, NIH (J. Battey)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.4

PROFESSIONAL:

2.4

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

A. Identification of receptors for GI peptides. We have recently discovered a specific high affinity receptor for the bombesin (Bn) related peptide neuromedin B (NMB) and developed specific ligands for it. In collaboration with T.W. Moody and T.H. Moran, these receptors have been identified to be widely distributed in the central nervous system. This receptor was cloned by J. Battey this year and from collaborative experiments pharmacologically characterized as distinct from the receptor for the mammalian bombesin-related peptide gastrin-releasing peptide (GRP). Because of the recent evidence that GI peptides may act as tumor growth factors we have investigated the occurrence of receptors for these on 10 human colon cell lines. We have demonstrated that of 12 different hormone and neurotransmitters examined, each human colon cancer cell line possesses receptors for at least one of these agents. The receptors were pharmacologically characterized and occupation by agents was shown to alter intracellular mediators. These studies are now being extended to determine whether occupation of these receptors affects tumor growth. In collaboration with R.H. Bell azaserine-induced neoplasms were shown to overexpress receptors for cholecystokinin (CCK). This finding may be important in the pathogenesis of these tumors because CCK-related peptides increase the rate of their development through an unknown process.

B. Characterization of receptors for gastrointestinal hormones by the development of specific antagonists. In collaborative studies with D.H. Coy we have extended our previous studies attempting to understand the active configuration of Bn related peptides to alter cellular function and to develop more potent antagonists. Our previous studies led us to propose that the COOH terminus of bombesin might adopt a β turn similar to proposed for LHRH and somatostatin. This conclusion has been supported during the year by molecular modeling studies done in collaboration by D.H. Coy as well as the synthesis of cyclic Bn analogues that are conformationally restricted and yet function as agonists and others as antagonists. Our previous showed that various des Met¹⁴ analogue of bombesin were extremely potent antagonists *in vitro* and *in vivo*. To improvement potency [D-Ala¹] Bn analogues were synthesized to decrease degradation and a pentafluoro phenylalanine group added to increase lipophilicity and the analogue [2,3,4,5,6 pentafluoro Phe⁶ D-Ala¹] Bn (6-13) methyl ester was found to have a duration of action *in vivo* of >10 fold that seen with other Bn receptor antagonists.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 53101-04 DDB

PERIOD COVERED

October 1, 1991, to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular basis of action of gastrointestinal peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R.T. Jensen	Branch Chief	DDB, NIDDK
Others:	S.A. Wank	Senior Investigator	DDB, NIDDK
	S.A. Mantey	Chemist	DDB, NIDDK
	J. Mrozinski, Jr.	Chemist	DDB, NIDDK
	C.P. Felley	Special Volunteer	DDB, NIDDK
	D.C. Metz	Senior Staff Fellow	DDB, NIDDK
	D.S. Yuan	Clinical Associate	DDB, NIDDK

COOPERATING UNITS (if any)

Department of Biochemistry, George Washington University (T.W. Moody); National Institute of Dental Research, Clinical Investigations Branch, NIH (R.J. Turner)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

A. We have recently discovered a specific receptor for the mammalian bombesin related peptide, Neuromedin B (NMB). In this study we have identified high affinity receptors on the rat glioblastoma tumor cell line, C-6 and we have investigated the cellular basis of action of peptides at this receptor in this cell line. The high affinity NMB receptors on C-6 cells pharmacologically were identical to those in normal tissues. NMB increased phospholipase C activity, increasing cellular calcium and phosphoinositides including IP_1 , IP_2 and IP_3 . No alterations in cAMP were seen. Similar results were found in collaboration with Dr. T.W. Moody (George Washington University) in human small cell lung cancer cells. These results show that both receptors for the mammalian Bn peptides NMB and GRP have similar cellular transduction mechanisms in that both activate phospholipase C.

B. The cellular basis of action of the structurally related peptides gastrin and cholecystokinin on stimulating pepsinogen release from chief cells is unclear and was investigated in the present study. Each peptide increased the mobilization of cellular calcium and increased IP_3 . However, they differed in their efficacy, stoichiometry and intracellular coupling. Detailed analysis demonstrated that these 2 peptides were altering cellular function by interacting with 2 distinct receptors with different coupling mechanisms.

C. To investigate the role of extracellular and intracellular calcium in secretagogue-induced enzyme secretion in pancreatic acini, we studied the ability of thapsigargin (TG), an inhibitor of microsomal calcium ATPase to alter secretagogue stimulated secretion and $[Ca^{2+}]_i$ levels. Our findings indicate that stimulation of enzyme secretion by secretagogues that increase IP_3 (1,4,5) does not depend on increases in $[Ca^{2+}]_i$ per se. In contrast, TG-induced potentiation of stimulation of secretagogues that increase cAMP results from increased free $[Ca^{2+}]_i$.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53200-02 DDB

PERIOD COVERED

October 1, 1991, to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Management of islet cell tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R.T. Jensen	Branch Chief	DDB, NIDDK
Others:	D.C. Metz	Senior Staff Fellow	DDB, NIDDK
	J.R. Pisegna	Clinical Associate	DDB, NIDDK
	V.A. Fishbeyn	Clinical Associate	DDB, NIDDK
	R.V. Benya	Clinical Associate	DDB, NIDDK
	D.B. Strader	Clinical Associate	DDB, NIDDK
	M. Orbuc	Clinical Associate	DDB, NIDDK

COOPERATING UNITS (if any)

National Cancer Institute, Surgery Branch, NIH (J.A. Norton, D. Fraker); Radiology Department, Clinical Center, NIH (J.L. Doppman)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Clinical Investigation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies in this section involve patients with islet cell tumors (primarily with Zollinger-Ellison syndrome [ZES]) and are directed at further understanding the pathogenesis of the syndrome and to developing alternative, more effective modes of therapy utilizing both medical and surgical approaches.

A. Control of symptoms due to hormone overproduction by tumor. Patients with ZES have islet cell tumors which release excess amounts of gastrin which causes a severe ulcer diathesis. During the year increasingly effective antiseecretory drugs, such as the H⁺-K⁺ ATPase inhibitor lansoprazole, has been shown to be effective and have a prolonged duration of action. Because many of these patients require surgery and cannot take oral drugs, a simplified effective method involving parenteral use of ranitidine has been developed. Currently used drugs such as omeprazole used at recommended doses cost many patients with ZES >\$10/day limiting compliance and during the year we have shown that currently recommended doses are too high and can be reduced resulting in significant financial savings without reducing efficacy.

B. Tumor localization and possible surgical cure. All patients with ZES have a potentially malignant tumor yet current cure rates are <20% because of their small size. We have developed increasingly effective means of localizing these tumors including portal venous sampling, intraarterial secretin administration with hepatic venous gastrin sampling, MRI scanning and careful exploration methods of the duodenum at surgery. In collaboration with J. Norton, NCI, we have increased the immediate post-op cure rate to 60% with a 5 year cure rate of 30%.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-53201-03 DDB

PERIOD COVERED

October 1, 1991, to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Receptors on gastric smooth muscle cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R.T. Jensen	Branch Chief	DDB, NIDDK
Others:	T. Pradhan	Chemist	DDB, NIDDK
	Z-F. Gu	Visiting Fellow	DDB, NIDDK
	Y. Kitsukawa	Visiting Fellow	DDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Gastroenterology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

A. Role of cAMP in mediating gastrointestinal smooth muscle relaxation by β -adrenergic agents or neuropeptides. It has been suggested but not proven that cAMP is an essential mediator of relaxation by these agents in GI smooth muscle. To investigate the role of cAMP in relaxation, in this study in dispersed gastric smooth muscle cells, the effect of the competitive protein kinase A antagonist (R_p -cAMPS) was investigated. The results demonstrated the activation of PKA is primarily responsible for mediating gastric smooth muscle relaxation produced by these agents.

B. Action of somatostatin on gastric smooth muscle. Somatostatin has potent effects on GI motility as well as gastric motility. To determine whether somatostatin can interact directly with GI smooth muscle cells, in this study the ability of various somatostatins to interact with gastric smooth muscle cells prepared from guinea pig stomach was examined. The results demonstrated smooth muscle cells possess high affinity somatostatin (SS) receptors. Smooth muscle cells rapidly degrade SS-14, but not SS-28 or a synthetic SS-8 analogue and this may be a major determinant of biologic activity. Somatostatin had no effect alone, but inhibited relaxants. The inhibitory effect was mediated both through a guanine nucleotide regulatory protein to inhibit adenylate cyclase and at a site distal to the generation of cAMP.

C. Characterization of opiod receptors on gastric smooth muscle. Numerous neural elements in the GI tract possess receptors for opiod peptides and some recent studies suggest that GI smooth muscle also possess opiod receptors. In this study specific opiod ligands as well as selective agonists and antagonists were used to study opiod receptors on gastric smooth muscle cells. The results demonstrated these GI smooth muscle cells possessed κ and μ opiod receptors but no δ receptors and that occupation of either the κ or μ receptor results in muscle contraction, therefore opiod peptides interacting with these receptors can possibly alter motility by interacting directly with smooth muscle cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53202-01 DDB

PERIOD COVERED

October 1, 1991, to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular characterization of receptors for GI peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S.A. Wank	Senior Investigator	DDB, NIDDK
Others:	R.T. Jensen	Branch Chief	DDB, NIDDK
	J.R. Pisegna	Clinical Associate	DDB, NIDDK
	A. de Weerth	Visiting Fellow	DDB, NIDDK
	T. Honda	Visiting Associate	DDB, NIDDK
	R.V. Benya	Clinical Associate	DDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1

PROFESSIONAL:

1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Receptors for gastrointestinal hormones are characterized using molecular biology methods. Methods include preparation of cDNAs, isolation and screening clones of interest, pharmacologic characterization of cloned receptors, screening for related receptor subtypes, *in situ* hybridization studies of the receptor of interest, chimeric receptor studies, preparation of antibodies to the isolated receptors of interest and characterization of genetic elements regulating receptor synthesis.

A. Cloning of the cholecystokinin (CCK) type A receptor (CCK_A receptor). At least two classes of receptors mediate the action of CCK and the structurally related peptide, gastrin in the CNS and in peripheral tissues; a CCK_A and a CCK_B/gastrin subtype. The CCK_A receptor mediates CCK effects in the CNS on satiety and peripheral physiologic effects such as gallbladder contraction and pancreatic secretion. In this study the CCK_A receptor was purified to homogeneity from rat pancreas and partial peptide sequencing was obtained after chemical/enzymatic digestion. Using degenerate oligonucleotide primers, clones were identified which led to identification of the nucleotide sequence of the rat pancreatic CCK_A receptor. The CCK_A receptor has 444 amino acids and seven putative transmembrane domains suggesting its membership in the guanine nucleotide-binding regulatory protein-coupled receptor superfamily. *In vitro* transcripts of the cDNA clone were functionally expressed in *Xenopus* oocytes and displayed the expected agonist and antagonist specificity.

B. Cloning of the CCK_B/gastrin receptor. The CCK_B/gastrin receptor is the principal receptor mediating the action of CCK on gastrointestinal motility, growth effects and is the predominant subtype in the CNS mediating such CNS effects as CCK-induced anxiety and analgesia. In this study using a CCK_A receptor cDNA probe, under low stringency hybridization conditions to screen a rat brain cDNA library and a cDNA library from AR42J cells, a pancreatic human cell line which possesses CCK_B receptors, a novel cDNA was identified. The cDNA sequence was identical in both tissues and encoded a 452 amino acid protein which has 48% identity to the CCK_A receptor. This receptor contains seven transmembrane domains characteristic of G-protein coupled receptors. COS-7 cells transfected with the novel cDNA demonstrated the pharmacology characteristic for a CCK_B/gastrin receptor subtype.

DEPARTMENT OF HEALTH AND HUMAN SERVICES-PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01 DK 53501-1 gDDB
PERIOD COVERED		
October 1, 1991 through September 30, 1992		
TITLE OF PROJECT(80 characters or less. Title must fit on one line between the borders.)		
Studies Relating to the Pathogenesis of Hepatic Encephalopathy and Fulminant Hepatic Failure		
PRINCIPAL INVESTIGATOR(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E. A. Jones	Chief
Others:	J. Vergalla	Chemist
	M. G. Swain	Guest Researcher
	C. Yurdaydin	Visiting Associate
	N.V. Bergasa	Senior Staff Fellow
		LDS, NIDDK
		LDS, NIDDK
		LDS, NIDDK
		LDS, NIDDK
		LDS, NIDDK
COOPERATING UNITS(If any)		
Laboratory of Neuroscience, NIDDK (P. Skolnick and A.S. Basile), Mental Health Intramural Research Program, NIMH (S.M. Paul), and National Institute of Drug Abuse, Baltimore (R.B. Rothman)		
LAB/BRANCH		
Digestive Diseases Branch		
SECTION		
Liver Diseases Section		
INSTITUTE AND LOCATION		
NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human Subjects	<input checked="" type="checkbox"/> (b) Human Tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK(Use standard unreduced type. Do not exceed the space provided.)		
<p>Both clinical and electrophysiologic (VER waveform) ameliorations of hepatic encephalopathy (HE) have been induced in animals with FHF by benzodiazepine (BZ) receptor ligands with antagonist properties. Furthermore, spontaneous in vitro activity of Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and, in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. In addition a BZ receptor antagonist reversed the hypersensitivity of HE rabbit neurons to depression by a GABA agonist. The functional status of the chloride ionophore of the GABA/BZ receptor complex has been shown to be normal in a rat model of HE due to FHF. Radioligand binding to BZ receptors, determined autoradiographically, was decreased in thin unwashed sections from HE rabbit brains. Purification and characterization of HE rat brain extracts revealed the presence of reversible, competitive, BZ receptor ligands with agonist properties. Two of these ligands have been chemically characterized as the 1,4-BZs diazepam and N-desmethyldiazepam. The concentrations of these compounds were 2-9 fold greater in HE rat brain than control brain. Overall, these findings suggest that in HE due to FHF: (i) There is increased GABA-ergic tone; (ii) Blockading of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) Endogenous BZ receptor agonists probably contribute to HE. The efficacy of BZ receptor ligands in ameliorating HE in animal models does not appear to depend on their intrinsic activity, but may be related to their affinity for BZ receptor subtypes in addition to the diazepam sensitive receptor.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES-PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK, 53503-16 DDB

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunologic Studies of Primary Biliary Cirrhosis

PRINCIPAL INVESTIGATOR(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E. A. Jones	Chief	LDS, NIDDK
Others:	J. Vergalla	Chemist	LDS, NIDDK
	N.V. Bergasa	Senior Staff Fellow	LDS, NIDDK
	M. Shindo	Guest Researcher	LDS, NIDDK
	R. Sallie	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS(IF ANY)

Division of Gastroenterology, University of Maryland at Baltimore (S.P. James)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.0

PROFESSIONAL

1.0

OTHER

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human Subjects ☒ (b) Human Tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK(Use standard unreduced type. Do not exceed the space provided.)

Primary biliary cirrhosis (PBC) appears to be a model autoimmune disease. Abnormal immune mechanisms are being studied in this disease, but so far a disease-specific immunologic deficit has not been defined with certainty. To determine whether previously described abnormalities of lymphocyte function in PBC might be due to altered function of immunoregulatory T cell subpopulations, phenotypic and functional characteristics of T cells that have the CD4 antigen detectable (by monoclonal antibody) on their surface were examined. Patients with PBC were found to have normal numbers of CD4+, Leu-8+ T cells, but, in contrast to patients with other liver diseases, suppression of immunoglobulin synthesis and mitogen-stimulated proliferation mediated by this subpopulation of T cells were defective. These defects in the function of CD4+, Leu-8+ T cells in patients with PBC were corrected by phorbol ester suggesting that abnormal function of the biochemical pathway involving protein kinase C may contribute to the immunological abnormalities exhibited by patients with PBC. In contrast to control patients with non-PBC chronic inflammatory liver disease, mRNA for IL-1,2,4,5 and 6, and TNF- α were not detected in liver biopsies from patients with PBC. mRNA for INF- γ was detected in 11 of 18 PBC liver biopsies. While these findings do not exclude a role for cytokines in the mediation of bile duct lesions in PBC, they suggest that immunologic injury that is not mediated by cytokines plays a major role in disease progression in PBC.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-53509 12 DDB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Natural History and Treatment of Chronic Type B Hepatitis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. (Name, title, laboratory, and institute affiliation))

PI:	Adrian M. Di Bisceglie	Chief	HSS, NIDDK
Others:	J.H. Hoofnagle	Director, DDN	NIDDK
	M.W. Fried	Med. Staff Fellow	HSS, NIDDK
	H. Conjeevaram	Med. Staff Fellow	HSS, NIDDK
	M. Beames	Lab Technician	HSS, NIDDK
	M. Shindo	Guest Researcher	HSS, NIDDK

COOPERATING UNITS (if any)

E. Tabor, DCE, NCI. S. Straus, LCI, NIAID. J. Gerin, Georgetown University.
 S.M. Feinstone, CBER, FDA

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

0

PROFESSIONAL

OTH/ILK

CHECK APPROPRIATE BOXES

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

This project has been transferred to DK Z01 53001-01 DDB,
 Hepatitis Studies Section.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201-DK-53510-11DDB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Natural History and Treatment of Chronic Hepatitis C

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator's Name, title, laboratory, and institute affiliation)

PI:	Adrian M. DiBisceglie	Chief	HSS, NIDDK
Others:	J.H. Hoofnagle	Director, DDN	NIDDK
	M.W. Fried	Med. Staff Fellow	HSS, NIDDK
	H. Conjeevaram	Med. Staff Fellow	HSS, NIDDK
	M. Beames	Lab Technician	HSS, NIDDK
	M. Shindo, L. Simpson	Guest Researchers	HSS, NIDDK
	K. Mahaney, E. Silva	Guest Researchers	HSS, NIDDK

COOPERATING UNITS (if any)

N.V. Bergasa, M.G. Swain, C. Yurdaydin LDS, NIDDK. H.J. Alter, DTM, CC, NIH.
J. Gerin, Georgetown University. S.M. Feinstone, CBER, FDA. K. Krawczynski,
Hepatitis Branch, CDC.

LAB/BRANCH

Digestive Diseases Branch

SECTION

Hepatitis Studies Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

PROFESSIONAL

0111LK

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

This project has been transferred to 201-DK-54002-01 DDB,
Hepatitis Studies Section.

DEPARTMENT OF HEALTH AND HUMAN SERVICES-PUBLIC HEALTH SERVICE NOTICE OF INTRANURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 53511-11 DDB
PERIOD COVERED October 1, 1991 through September 30, 1992	
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Trials of Therapies for Primary Biliary Cirrhosis	
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)	
PI: E. A. Jones	Chief LDS, NIDDK
Others: J.H. Hoofnagle	Director DDDN, NIDDK
N.V. Bergasa	Senior Staff Fellow LDS, NIDDK
M.G. Swain	Guest Researcher LDS, NIDDK
C. Yurdaydin	Medical Staff Fellow LDS, NIDDK
S.C. Chia	Medical Staff Fellow LDS, NIDDK
R. Sallie	Medical Staff Fellow LDS, NIDDK
COOPERATING UNITS (IF ANY)	
AB/BRANCH Digestive Diseases Branch	
SECTION Liver Diseases Section	
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892	
Total Non-Veers: 1	PROFESSIONAL: 1
OTHER:	
CHECK APPROPRIATE BOX(ES)	
<input checked="" type="checkbox"/> (a) Human Subjects	<input checked="" type="checkbox"/> (b) Human Tissues
<input type="checkbox"/> (a1) Minors	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a2) Interviews	
SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)	
<p>Primary biliary cirrhosis (PBC) is an autoimmune disease of unknown etiology characterized by slowly progressive intrahepatic cholestasis due to non-suppurative inflammatory destruction of small intrahepatic bile ducts. Because some other autoimmune diseases appear to respond favorably to certain immunosuppressive agents, trials of selected immunosuppressive drugs are being undertaken in patients with PBC. One promising drug of this type is methotrexate. Ten patients with symptomatic PBC were admitted to an open trial of low-dose (15 mg) once weekly oral methotrexate. Two patients with advanced (stage III-IV) disease dropped out after 4-5 months. The remaining 8 have been followed for up to 36 months. Methotrexate therapy in these patients was associated with significant decreases in elevated serum levels of IgM and alkaline phosphatase and a more than 50% decrease in symptom scores for fatigue and pruritus. Serum ALT levels initially increased (at 4 and 8 mo.) but the mean subsequently fell to 10% below baseline. In 8 patients, who had liver biopsies before and after one year of treatment, methotrexate was associated with a substantial decrease in hepatic inflammation but no obvious change in fibrosis. Patients with Stage III disease progressed during the second year of treatment and methotrexate was discontinued in 3 of these patients. A randomized trial of two doses of methotrexate (7.5 and 15 mg/week) for asymptomatic as well as symptomatic patients with PBC has been initiated. 25 patients have been admitted to this trial.</p>	

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201-DK-53514-07

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunological Studies in Chronic Viral Hepatitis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. (Name, title, laboratory, and present affiliation.)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director	DDN
	A. Di Bisceglie	Visiting Scientist	LDS, NIDDK
	M. Shindo	Guest Researcher	LDS, NIDDK

COOPERATING UNITS (if any)

Division of Gastroenterology, University of Maryland at Baltimore (S.P. James)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

0

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

This project has been terminated.

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Opiate System in Cholestatic Liver Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E. A. Jones	Chief	
Others:	N. V. Bergasa	Senior Staff Fellow	LDS, NIDDK
	J. Vergalla	Chemist	LDS, NIDDK
	M.G. Swain	Medical Staff Fellow	LDS, NIDDK
	C. Yurdaydin	Medical Staff Fellow	LDS, NIDDK
	R. Sallie	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS (IF ANY) NIDA Research Center, Baltimore (R.B. Rothman)
NHLBI, Laboratory of Biochemical Genetics (S. Sabol)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2

PROFESSIONAL

1.5

OTHER

5

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human Subjects ☒ (b) Human Tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The hypothesis that increased functional activity of the opioid system contributes to the pathophysiology of cholestasis is being tested in animal models and man. Using an objective monitoring system naloxone infusions have been shown to decrease scratching activity independent of gross body movements by 26-96% in 8 patients with pruritus due to primary biliary cirrhosis in a single blinded controlled-trial. In a double blind controlled trial naloxone infusions were associated with a decrease in scratching activity in 24 of 29 patients with pruritus due to chronic cholestasis ($p < 0.003$). These findings confirm a role for the opioid system in the pathogenesis of the pruritus of cholestasis and suggest that not only opiates, like morphine, but also endogenous opioids mediate pruritus. The ability of the orally bioavailable opiate antagonist nalmefene to provide long-term control of the pruritus of chronic cholestasis is being evaluated. An open label study in 16 patients has been completed with encouraging results and a double-blind placebo-controlled study has been initiated. Increased opiate tone in cholestasis is strongly suggested by antinociception that is stereoselectively reversed by naloxone and down regulation of brain mu-opioid receptors in bile duct resected (BDR) rats, but not rats with acute hepatocellular necrosis. Using Northern blots preproenkephalin mRNA has been shown to be unequivocally expressed in livers of BDR rats but not controls. This expression was shown by hybridization histochemistry to be in the nuclei of proliferating bile ductular cells and was associated with positive immunohistochemical staining for met-enkephalin. These findings indicate that the fetal ability of the liver to synthesize opioid peptides is regained during cholestasis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

DK Z01 54001 01

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Natural History and Treatment of Chronic Hepatitis B

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Adrian M. Di Bisceglie	Chief	HSS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	M.W. Fried	Medical Staff Fellow	HSS, NIDDK
	H. Conjeevaram	Medical Staff Fellow	HSS, NIDDK
	M. Beames	Lab. Technician	HSS, NIDDK
	M. Shindo	Guest Researcher	HSS, NIDDK

COOPERATING INSTITUTES (if any)

E. Tabor, DCE, NCI. S. Straus, LCI, NIAID. J. Gerin, Georgetown University. S.M. Feinstone, CBER, FDA

LAB/BRANCH

Digestive Diseases Branch

SECTION

Hepatitis Studies Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

3.75

PROFESSIONAL:

3

OTHER

.75

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Patients with chronic type B hepatitis are being evaluated and followed prospectively to determine the long-term natural history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials of antiviral or immunomodulatory agents. Several agents have been evaluated including alpha interferon, ribavirin, and fluoro-iodo arabinofuranosyl-uracil (FIAU). Alpha interferon continues to be evaluated in atypical or unusual patients who would ordinarily be excluded from controlled trials including those with decompensated cirrhosis, extra-hepatic complications of hepatitis B, atypical serologic markers and children with hepatitis B.

A pilot study of ribavirin in 18 patients with chronic hepatitis B found that both serum HBV DNA and alanine aminotransferase (ALT) levels decreased significantly within 4 weeks of starting therapy. The decrease in mean HBV DNA averaged 23%. Mean ALT levels decreased by 40%, becoming normal in 4 patients at the end of treatment. However, these changes were transient only. Only 1 patient lost hepatitis B e antigen from serum during therapy. Subsequently, HBeAg reappeared and HBV DNA again became detectable in this individual.

A pilot study of FIAU, a nucleoside analogue, is currently underway. Nine patients have completed 4 weeks of therapy and have been followed for at least 1 week after stopping FIAU. At all doses of FIAU tested so far (0.05, 0.1 and 0.25 mg/kg/day), therapy is associated with marked suppression (85% decrease) of serum HBV DNA levels after 4 weeks of therapy. FIAU appears to be well-tolerated with few side effects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

DK Z01 54002 01

PERIOD COVERED

October 1, 1991 through September 30, 1992.

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Studies of the Natural History and Treatment of Chronic Hepatitis C

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Adrian M. Di Bisceglie	Chief	HSS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	M.W. Fried	Medical Staff Fellow	HSS, NIDDK
	H. Conjeevaram	Medical Staff Fellow	HSS, NIDDK
	M. Beames,	Lab. Technician	HSS, NIDDK
	M. Shindo, L. Simpson	Guest Researchers	HSS, NIDDK
	K. Mahaney, E. Silva	Guest Researchers	HSS, NIDDK

COOPERATING UNITS (if any)

N.V. Bergasa, M.G. Swain, C. Yurdaydin LDS, NIDDK. H.J. Alter, DTM, CC, NIH. J. Gerin, Georgetown University. S.M. Feinstone, CBER, FDA. K. Krawczynski, Hepatitis Branch, CDC

LAB/BRANCH

Digestive Diseases Branch

SECTION

Hepatitis Studies Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS

5.25

PROFESSIONAL

4.25

OTHER

1

CHECK APPROPRIATE BOXES:

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

Patients with well-documented chronic hepatitis C are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients are available to evaluate experimental therapies. Previous studies have shown that alpha interferon therapy has a short-term beneficial effect in approximately 50% of patients during treatment and that the beneficial response is sustained in approximately 20% after stopping interferon. A pilot study of ribavirin therapy indicated that this agent has an antiviral effect against hepatitis C. A randomized, placebo-controlled trial of ribavirin is currently underway.

ANNUAL REPORT OF THE MOLECULAR AND CELLULAR ENDOCRINOLOGY BRANCH

(formerly, the MOLECULAR, CELLULAR AND NUTRITIONAL ENDOCRINOLOGY BRANCH)

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The MCEB continues basic and clinical investigations in the areas of molecular regulation and neuroendocrinology (Molecular Regulation and Neuroendocrinology Section, Bruce D. Weintraub, Chief); and growth and development (Growth and Development Section, Matthew M. Rechler, Chief). The Branch has had many visiting fellows and associates, as well as international collaborations with the University of Milan, Italy; University of Marseilles, France; Karolinska Institute, Sweden; Postgraduate School of Obstetrics and Gynecology, University of Auckland, New Zealand; University of Naples, Italy; Department of Medicine, University of Gothenburg, Sweden; University of Madrid, Spain. In the past year Dr. Weintraub was honored as a Visiting Professor at Beth Israel Hospital, Harvard Medical School as the second Sydney Ingbar Lecturer. He was also a plenary speaker at the International Thyroid Meeting in Amsterdam, the UCLA Symposium on Thyroid Hormone Action in Tamaron, Colorado, The Endocrine Society Meeting in San Antonio, Texas, the Annual Meeting of the Maryland Endocrine Society, as well as several other national meetings.

I. THYROTROPIN, THYROTROPIN-RELEASING HORMONE AND THYROID HORMONE: MOLECULAR BIOLOGY, REGULATION, ACTION, AND PATHOPHYSIOLOGY.

A. Small Scale and Large Scale Production of Recombinant Human Thyrotropin (TSH) and Its Analogues: Clinical Trials in Patients with Thyroid Cancer

Previous studies by our laboratory have shown that recombinant hTSH produced by transient transfection of human embryonal kidney (293) cells was bioactive. However, the level of transient expression was not sufficient to carry out further characterization. In order to obtain high level expression of rhTSH, the original TSH β minigene (2kb) was modified. A 989bp hTSH β minigene was synthesized and was inserted into two transient expression vectors by directional cloning.

Cos-7 and 293 cells were cotransfected with plasmids containing an hTSH β minigene driven by either simian virus (SV40) late promoter or cytomegalovirus (CMV) promoter, and a plasmid pAV₂.hCGacDNA (621bp) driven by SV40 early promoter. Immunoactivity of transiently expressed rhTSH in media was determined by immunoradiometric assay, and bioactivity was measured by its ability to incorporate ³H thymidine as well as to stimulate cAMP production in rat thyroid FRTL5 cells. Both the cell types Cos-7 and 293 express transiently high levels of immunoactive rhTSH (150.2 \pm 5.4 μ U/ml) when the CMV promoter containing expression vector and a ratio of a subunit cDNA to TSH β 3:1 was used. Excellent correlation was observed between immuno- and bio-activity when transiently expressed rhTSH was measured in

concentrated media from transfected cells. Production of biologically active hTSH in transient expression assay can be used as a very useful screening method for the initial characterization of mutants and variants of recombinant TSH, prior to stable transfection, as described below.

We have previously characterized recombinant human TSH (rhTSH) produced by Chinese hamster ovary cells attached to microcarrier beads in a large-scale bioreactor after stable transfection of hCG α and hTSH β minigenes. We have now produced in our laboratory greater than 200 mg of rhTSH using a small hollow-fiber bioreactor system. The daily production rate of rhTSH (0.5-3 mg) in this system was affected by various factors, including concentrations of glucose, lactate, serum, as well as proteolytic activity deriving from cells.

In collaboration with the Biotechnology Unit of NIDDK, we have used immunoaffinity chromatography or cation-exchange and dye affinity chromatography to purify the rhTSH for further analysis. We observed that the rhTSH produced in the hollow-fiber bioreactor showed 3-fold higher bioactivity *in vitro* than commercially produced rhTSH. In addition, we found about 4-5-fold lower sialic acid content in rhTSH produced in the hollow-fiber bioreactor than in the rhTSH produced on a large scale. rhTSH produced in the hollow-fiber bioreactor, large-scale produced rhTSH, as well as pituitary hTSH have been examined by chromatofocusing on a Mono P column with a linear pH gradient from 7.1 to 4.0 and 8.3 to 5.0. On chromatofocusing, rhTSH was separated into at least 5 isoforms of pI range 7.1-5.5, whereas pituitary hTSH (I-7) was separated into 6 isoforms with pI generally more acidic than both rhTSH preparations. The bioactivities of the isoforms were determined in 3 different FRTL-5 cell bioassays (cAMP, growth, and deiodinase activity). More acidic fractions of rhTSH (pI 6.5-5.5) required 2-3 fold higher concentration for half-maximal stimulation of cAMP production than the more basic fractions (pI 7.1-6.9). Essentially similar results were obtained in 2 other *in vitro* assays: growth and deiodinase activity assays. Carbohydrate compositional analysis of the fractions showed higher sialic acid content in more acidic rhTSH fractions. Pituitary hTSH acidic isoforms (pI 5.75-4.75) showed higher total sulfate and sialic acid content than more basic fractions (pI 6.5-5.75). No major differences in neutral oligosaccharides between fractions were found. Since it is known that sialylation alters the metabolic clearance of glycoproteins, we examined the clearance rate of rhTSH isoforms in rats. We found that the clearance rate is inversely related to the degree of sialylation, from the highly sialylated rhTSH isoform with pI 6.3, which has the slowest clearance rate, to the asialo-rhTSH, which has the most rapid clearance rate.

We also measured the stimulation of unlabeled thyroxine levels in mice by rhTSH preparations using a novel *in vivo* bioassay procedure recently developed in our laboratory. We found that, highly sialylated, unfractionated, large-scale produced recombinant TSH-G is more active *in vivo* than the less sialylated recombinant TSH produced in the hollow-fiber bioreactor. We have also found that the most sialylated isoform is the most active preparation *in vivo*.

Through a Cooperative Research and Development Agreement with the Genzyme Corporation (Boston), recombinant human TSH is being used for clinical studies in patients with thyroid cancer. This product is expected to stimulate uptake of radioactive iodide for both diagnostic and therapeutic purposes and to obviate the need for performing uptake studies in hypothyroid patients. An Investigational New Drug Application has been approved by the Food and Drug Administration and phase 1-2 clinical trials have been completed at NIH and four other medical centers showing preliminary efficacy and safety in 19 patients. Phase 3 trials are being planned.

... N. R. Thotakura, L. Joshi, M. Szkudlinski, J. E. Palmer, B. D. Weintraub

B. Sequential enzymatic deglycosylation of recombinant hTSH and the role of individual monosaccharides in the oligosaccharide chains of TSH in the hormone's action:

Recombinant (r)hTSH, expressed in Chinese hamster ovary cells contains sialic acid-terminating oligosaccharides as opposed to the presence of terminal sulfate residues in addition to the sialic acids in native pituitary hTSH. Sequential deglycosylation of hTSH to study the role of non-terminal saccharides was not previously possible due to the lack of suitable sulfatase preparations, first to remove the terminal sulfate. Now, using rhTSH, we have removed the monosaccharides sequentially by digestion with several exoglycosidases: neuraminidase, β -galactosidase, β -N-acetyl glucosaminidase and α - and β -mannosidases. The effect of removal of each of the sugars on the receptor-binding activity, and the *in vitro* and *in vivo* bioactivities of TSH is currently being studied. Prior studies have shown that desialylated rhTSH is 10 to 20-fold more active than the native hormone in *in vitro* bioassays, but is rapidly cleared from the circulation and thus has very low *in vivo* bioactivity. We have now shown that removal of galactose and N-acetyl glucosamine do not significantly reduce the *in vitro* bioactivity compared to the desialylated derivative. Therefore, the *in vivo* bioactivity of these derivatives may possibly be higher than the native hormone if their metabolic clearance rates are lower than the desialylated TSH

... N. R. Thotakura, M. W. Szkudlinski, B. D. Weintraub

C. Transcriptional regulation of human TSH β (hTSH β) gene expression

The hTSH β gene is expressed only in the thyrotrophs of the anterior pituitary, where its expression is induced by TRH, MA or cAMP and inhibited by the thyroid hormone. Our previous studies indicated that the hormonal induction of the hTSH β gene requires the pituitary-specific factor pit-1 which binds to the -128/-61 region, while the thyroid hormone inhibition is mediated by thyroid hormone receptor binding to the sequences in the first exon.

In this study, we found that the interaction of Pit-1 with AP-1-like ubiquitous factor induced by PMA or cAMP is crucial for the activation of the hTSH β gene. Detailed mutational analysis in the gel mobility shift assays revealed that the AP-1-like ubiquitous factor binds to the TGGGTCA sequences at -1/+6 of the hTSH β promoter which differ in only one nucleotide from the consensus AP-1 site (TGAGTCA). Mutation of this element alone abolished the induction by Pit-1/PMA or Pit-1/cAMP completely even in the presence of Pit-1 indicating an interaction between the AP-1-like factor and Pit-1. However, Pit-1 neither increased the binding of the AP-1-like factor to its target sequences nor associated with this factor to form a heterodimer. Since the TGGGTCA element which mediates both the PMA and cAMP responsiveness of the hTSH β gene overlaps with the known thyroid hormone inhibitory element, it appears that different signal transduction pathways and regulation by hormone receptors converge to the same element in the hTSH β gene.

We have noted that an AP-1 binding site from -1 to +6 bp overlaps with domain 1 in the first exon of hTSH β and examined possible interactions between c-fos and c-jun, the heterodimeric constituents of AP-1, with thyroid hormone receptor in this region. Transient transfections were performed in human placental cells (JEG3) using luciferase expression vectors containing transversion mutations in the first exon of a -125 to +37 fragment of the hTSH β gene.

Cotransfection of expression plasmids for c-jun and c-fos alone increased basal expression of native hTSH β 3.7 and 7.3-fold, respectively and together 11-fold. Stimulation of transversion mutations at +2 bp and +4 bp by cotransfected c-jun was 244% and 18% and for c-fos and 185% and 30%, respectively of of wild type stimulation. The transversion mutations alone reduced T3 inhibition by 26% and 29% respectively. T3 inhibition of the wild type construct from 19% to 57%, and of the transversion mutations at +2 and +4 bp to 74% and 59% respectively. Cotransfection of equal amounts of c-fos and c-jun resulted in a 40% inhibition, partially reversing the c-fos-induced increased inhibition. Thus, c-fos and c-jun are potent stimulators of hTSH β expression in opposite directions. Control of the relative cellular levels of these two proto-oncogenes may thus play a major role in modulating thyroid hormone inhibitory responses.

We have recently optimized a primary anterior pituitary cell culture system to reexamine the positive and negative regulation of TSH β transcription in thyrotropes. Enzymatic dispersion of normal rat anterior pituitary tissue yields cells for primary monolayer culture. Transient transfection using calcium phosphate precipitation of hTSH β constructs that were placed within a chloramphenicol acetyl transferase (CAT) reporter were demonstrated to increase CAT activity 2-3 fold by TRH, 15- to 20-fold increase by forskolin, and a 50-70% reduction in both basal and forskolin-stimulated CAT activity by thyroid hormone. Previous work with GH3 cells has demonstrated at most a 30% reduction in gene expression by T3. Mutations from the 3' end of the first exon of the hTSH β gene (-128/+37CAT, -128/+8CAT, and -128/+2CAT) which abolish all of the most 3' thyroid hormone response element (TRE) or part of the most 5' TRE in the first exon show a diminution of inhibition by T3. The remaining two bases (GG=+1+2) of the first TRE may be sufficient to support T3 inhibition. To determine if one or both bases are required for T3 inhibition, scanning mutational analysis using transient transfection of hTSH β gene expression vectors containing 5 bp cassette mutations within the first exon will be performed.

... M. K. Kim, D. L. Bodenner, L. A. Lesoon-Wood, B. D. Weintraub

D. Mutant β 1 T3-Receptors from Kindreds with Generalized Resistance to Thyroid Hormone.

Generalized resistance to thyroid hormone (GRTH) is an inherited disease linked to mutations in the β T3 receptor (hTR β) gene and characterized clinically by the resistance of peripheral and pituitary tissues to the action of thyroid hormone. Of the over 30 different mutations identified in the ligand binding domain of the hTR β 1, several were shown to inhibit normal hTR function by a dominant negative mechanism, which is likely to mediate the phenotype of this disease. Three different mechanisms have been proposed to mediate the inhibitory action of mutant hTR β 1: (1) competition for binding to T3-response elements (TRE), (2) competition for limiting amounts of TR auxiliary proteins, (3) formation of inactive dimers between normal and mutant receptors. Indirect evidence in HeLa cells has been presented to suggest that the last mechanism is unlikely to be important, while the former two mechanisms are likely to occur. Therefore, we investigated directly the DNA-binding properties of three different mutant hTR β 1 proteins derived from *in vitro* translation (ED, OK, PV) in gel-shift (TREpal probe) and avidin-biotin complex DNA binding (ABCD, rGH -186 to -158 bp probe) assays. Since RXR β was recently shown to be the predominant TR auxiliary protein in HeLa cells, we also investigated the interaction of the mutant hTR β 1 with RXR β . All the mutant receptors bound well to both the TREpal and the rGH-TRE. In gel-shift experiments, the apparent dissociation rates of mutant hTR β 1 were not different from normal hTR β 1 (7 min for 50% dissociation at 37°C). Similarly, the binding avidity of mutant receptors to rGH-TRE in the ABCD assay was not different from

wild-type at various DNA and T3 concentrations. The addition of *in vitro* translated RXR β induced the formation of heterodimers with normal and mutant hTR β 1 in gel shift experiments. These results suggest that the interaction of these mutant hTR β 1 with auxiliary proteins is intact and therefore competition for limiting amounts of accessory factors might be involved in mediating the dominant negative effect. To test this hypothesis directly, we transiently transfected HeLa cells with a pMTV-TREpal-CAT reporter and the appropriate pSV2 expression vectors. Although the dominant negative potency of the mutant hTR β 1 and hRAR in a dose-dependent manner, the dominant negative potency of the mutant hTR β 1 on normal hTR β 1 function could not be reversed under the conditions tested. In conclusion, we have demonstrated that hTR β 1 from different kindreds with GRTH bind normally to DNA and RXR β , despite the presence of mutations in a domain that is involved in modulating these interactions. Since transfection of excess amounts of RXR β did not reverse the inhibitory potency of mutant hTR β 1, we propose that competition of mutant and normal hTR β 1 for DNA-binding rather than for limiting amounts of RXR β is involved in mediating the dominant negative action of the mutant hTR β 1.

We have recently investigated whether the heterogeneous phenotypic features that occur not only among kindreds but also within the same kindred might be due to the expression of different ratios of mutant and normal receptors in tissues. Using an allele-specific primer extension method, we determined the relative expression of normal and mutant mRNAs from the fibroblasts of affected and unaffected members of two kindreds with GRTH. While 3 affected members of this kindred, as expected, had nearly equal amounts of normal and mutant hTR β RNA, two affected members had mutant mRNA levels that accounted for at least 70% of the hTR β mRNA. When several clinical parameters of GRTH were compared in these affected members, we found that the cases exhibiting a high mutant to normal hTR β ratio had considerably more bone resistance and growth retardation. Furthermore, one affected case relative mutant's expression decrease from 84% to 67% as her growth curve increased from less than the 5% to the 25%. The second kindred who did not vary significantly from the expected 50 percentile did not have bone resistance. These results suggest that differing ratios of normal and mutant hTR receptors may be age and growth related and may account for the reported attenuation of phenotypic symptoms with age in these patients.

We have also investigated the relationship between generalized resistance to thyroid hormone (GRTH) and attention deficit hyperactivity disorder (ADHD). Eighteen unrelated kindreds with GRTH were included in the initial study; in 13 of these, distinct mutations in the human thyroid receptor β (hTR β) gene were located in exons 9 and 10 which codes for the ligand-binding domain of the receptor. The study population consisted of 49 affected and 55 unaffected family members comprising a total of 52 adults (≥ 18 yrs) and 52 children (< 18 yrs). Patients were evaluated by interviewers blind with respect to the diagnosis of GRTH using structured psychiatric questionnaires. 50% (11/22) of GRTH affected adults compared to 7% (2/30) of unaffected adult family members ($p < 0.0001$), and 70% (19/27) of GRTH affected children compared to 20% (5/25) of unaffected child family members ($p < 0.0001$) met criteria for the diagnosis of ADHD. Further, the mean ADHD symptom score was 2.5-fold higher in the affected patient group than the group of unaffected family members (7.0 vs. 2.89, $p < 0.0001$). Other psychiatric diagnoses were not significantly different between the two groups. The strong association of ADHD to GRTH could not be explained by elevated thyroid hormone levels in patients with GRTH. Anecdotal evidence showed that symptoms of ADHD were ameliorated with liothyronine replacement therapy in 12 of 19 patients, suggesting that the behavioral

manifestations of a defective thyroid hormone receptor may be partially reversed by liothyronine treatment.

Another aspect of this study investigated the effect of GRTH on cerebral metabolism determined by positron emission tomography (PET). PET brain scans, using [18F]fluoro-2-deoxy-D-glucose, were obtained on nine adult patients with GRTH and nine age and sex-matched normal controls while they performed an auditory continuous-performance task (CPT) designed to activate areas of the brain necessary for sustained attention. The auditory CPT performance score was significantly lower in 9 GRTH patients who had PET scans than the age and sex-matched controls (mean score; 148 vs. 87, $p < 0.03$). Of the 60 regions of interest measured by PET, glucose metabolic rates of GRTH patients were significantly decreased in the superior right parietal lobe ($p < 0.05$) and increased in the mid-occipital lobes ($p < 0.01$) and the right thalamus ($p < 0.05$). The PET data suggest that GRTH may cause decreased metabolism in an area of the brain (right parietal lobe) that subserves sustained attention.

These data demonstrate that ADHD is strongly and specifically associated with GRTH. This is the first defined molecular model of ADHD and may provide new insights into the basic pathogenesis of this disorder.

.... A. J. Mixson, C. H. Meier, P. Hauser, B. D. Weintraub

II. INSULIN-LIKE GROWTH FACTORS

We have extended our studies to understand how insulin-like growth factor binding proteins (IGFBPs) modulate the biological action of IGF-I and IGF-II. The IGFs occur in plasma, other extracellular fluids, and tissues complexed to one or more members of the family of 6 IGFBPs. The IGFBPs determine the bioavailability of the IGFs, and may inhibit or potentiate their actions. A major determinant of the biological activity of the IGFBPs is its abundance in different tissues, which is determined in part by regulation of its synthesis. We have studied the regulation of IGFBP-2 transcription in rat liver by fasting, the regulation of IGFBP-1 transcription by insulin in diabetes and in rat hepatoma cells, and demonstrated that IGFBP-6 in human cerebrospinal fluid is O-glycosylated. (1) IGFBP-2 mRNA is increased in fasted rat liver (but not brain or kidney), and rapidly normalized by refeeding. Run-on transcription studied demonstrated a corresponding increase in the initiation of transcription of the IGFBP-2 gene. IGFBP-2 transcription and IGFBP-2 mRNA also are increased in neonatal rat liver. (2) Insulin rapidly inhibits IGFBP-1 transcription and IGFBP-1 mRNA in diabetic rat liver. Similarly, in H4-II-E rat hepatoma cells, insulin decreased IGFBP-1 transcription within 20 min. This is a direct effect of insulin, and is mediated by the insulin receptor. The H4-II-E cell line will be used to identify the insulin response elements in the IGFBP-1 promoter. (3) IGFBP-6, one of the predominant IGFBPs in human cerebrospinal fluid, was purified and shown to be O-glycosylated. Enzymatic deglycosylation did not alter its high, preferential affinity for IGF-II versus IGF-I. The effect of O-linked oligosaccharides on the localization of IGFBP-6 in tissues and its biological activity remain to be determined.

.... M. M. Rechler, L. A. Bach, Y. Boisclair, A. L. Brown, G. T. Ooi, D. S. Suh, L.Y.-H. Tseng, Y. W.-H. Yang

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER ZO1 DK 55000-20 MCEB			
PERIOD COVERED October 1, 1991 to September 30, 1992					
TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.) Biosynthesis, Glycosylation, and Action of Thyrotropin: Clinical Trials of Recombinant TSH					
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; vertical-align: top;"> PI: B. D. Weintraub Others: N. R. Thotakura J. E. Palmer M. Szkudlinski L. Joshi </td> <td style="width: 40%; vertical-align: top;"> Chief Visiting Scientist Biologist Visiting Fellow Senior Staff Fellow </td> <td style="width: 30%; vertical-align: top;"> MCEB, NIDDK MCEB, NIDDK MCEB, NIDDK MCEB, NIDDK MCEB, NIDDK </td> </tr> </table>			PI: B. D. Weintraub Others: N. R. Thotakura J. E. Palmer M. Szkudlinski L. Joshi	Chief Visiting Scientist Biologist Visiting Fellow Senior Staff Fellow	MCEB, NIDDK MCEB, NIDDK MCEB, NIDDK MCEB, NIDDK MCEB, NIDDK
PI: B. D. Weintraub Others: N. R. Thotakura J. E. Palmer M. Szkudlinski L. Joshi	Chief Visiting Scientist Biologist Visiting Fellow Senior Staff Fellow	MCEB, NIDDK MCEB, NIDDK MCEB, NIDDK MCEB, NIDDK MCEB, NIDDK			
COOPERATING UNITS (if any) None					
LAB/BRANCH Molecular and Cellular Endocrinology Branch					
SECTION Molecular Regulation and Neuroendocrinology Section					
INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, Maryland 20892					
TOTAL MAN-YEARS: 3.5	PROFESSIONAL: 3.5	OTHER: 0			
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews					
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Recombinant thyrotropin has been produced by novel methods of transient or stable transfection of alpha and hTSH beta minigenes into Chinese hamster ovary cells. Utilizing products produced in a large scale bioreactor as well as a hollow fiber bioreactor, we have been able to purify several hundred milligrams of this product. In general, the recombinant product is more sialylated than standard pituitary TSH and consists of at least 7 isoforms differing primarily in sialic acid content. The more sialylated forms have less in vitro biologic activity, but a longer metabolic clearance rate and enhanced in vivo biologic activity. Various analogs of TSH have also been produced by modifying various regions of the beta subunit of TSH. Such analogs appear to have different in vitro as well as in vivo biologic activity. The role of specific carbohydrate chains in the action of TSH has been studied by sequential enzymatic deglycosylation of recombinant TSH followed by characterization in in vitro and in vivo bioassays.</p> <p>Through a Cooperative Research and Development Agreement with the Genzyme Corporation (Boston), recombinant human TSH is being used for clinical studies in patients with thyroid cancer. This product is expected to stimulate uptake of radioactive iodine for both diagnostic and therapeutic purposes and to obviate the need for performing uptake studies in hypothyroid patients. An Investigational New Drug Application has been approved by the Food and Drug Administration and phase I and II clinical trials have been completed at NIH and 4 other medical centers showing preliminary efficacy and safety in 19 patients. Phase III trials are currently being planned.</p>					

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
ZO1 DK 55002-12 MCEB

PERIOD COVERED
October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)
Molecular Biology of Pituitary Glycoprotein Hormones and Hypothalamic Releasing Hormones

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B. D. Weintraub	Chief	MCEB, NIDDK
Others:	D. L. Bodenner	Senior Staff Fellow	MCEB, NIDDK
	Myung K. Kim	Senior Staff Fellow	MCEB, NIDDK
	L. A. Lesoon-Wood	IRTA Postdoctoral Fellow	MCEB, NIDDK

COOPERATING UNITS (if any)
Dr. Fredric E. Wondisford, Case Western Reserve School of Medicine, Cleveland, Ohio

LAB/BRANCH
Molecular and Cellular Endocrinology Branch

SECTION
Molecular Regulation and Neuroendocrinology Section

INSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.5

PROFESSIONAL: 2.5

OTHER: 0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

The human TSH beta gene is expressed only in the thyrotrophs of the anterior pituitary where it is regulated by hypothalamic hormones, cyclic AMP as well as thyroid hormone. Recent studies have indicated that TRH and cyclic AMP mediate their transcriptional action in large part through changes in phosphorylation of the specific pituitary factor pit-1 which binds to a region upstream of the start of transcription. In contrast, thyroid hormone mediates its inhibition through binding to a regulatory element downstream of the start of transcription in the first untranslated exon.

Recent studies have indicated that the thyroid hormone receptor interacts with other factors in the mediation of hormone action. Such factors include the proto-oncogenes c-fos and c-jun which are the heterodimeric constituents of AP-1. Various transcriptional studies have indicated that c-fos augments thyroid hormone induced inhibition of transcription, whereas c-jun blunts such effect. Thus, control of the relative cellular levels of these 2 proto-oncogenes may play a major role in modulating thyroid hormone inhibitory responses.

We have recently optimized a primary anterior pituitary cell culture system to reexamine the positive and negative regulation of human TSH beta transcription in native thyrotrophs. Such cells show general similarities but certain differences compared to previously transfected permanent cell lines such as GH3. These differences may be attributed to unique thyrotropic transcription factors which are currently being elucidated.

NOTICE OF INTRAMURAL RESEARCH
PROJECTNUMBER
Z01DK55006-19MCE

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin-like Growth Factors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M.M. Rechler	Chief, GDS	MCEB, NIDDK
Others:	L.A. Bach	Visiting Associate	MCEB, NIDDK
	Y. Boisclair	Special Volunteer	MCEB, NIDDK
	A.L. Brown	Sr. Staff Fellow	MCEB, NIDDK
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	D.S. Suh	Special Volunteer	MCEB, NIDDK
	L.Y.-H. Tseng	Chemist	MCEB, NIDDK
	Y.W.-H. Yang	Sr. Staff Fellow	MCEB, NIDDK

COOPERATING UNITS (if any)

University of California, Riverside (D.S. Straus); MRN MCEB NIDDK (N.R. Thotakura)

LAB/BRANCH

Molecular & Cellular Endocrinology Branch

SECTION

Growth & Development Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

7.5

PROFESSIONAL:

6.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We have extended our studies designed to understand how insulin-like growth factor binding proteins (IGFBPs) modulate the biological actions of IGF-I and IGF-II. The IGFs occur in plasma, other extracellular fluids, and tissues complexed to one or more members of the family of 6 IGFBPs. The IGFBPs determine the bioavailability of the IGFs, and may inhibit or potentiate their actions. A major determinant of the biological activity of the IGFBPs is its abundance in different tissues, which is determined in part by regulation of its synthesis. We have studied the regulation of IGFBP-2 transcription in rat liver by fasting, the regulation of IGFBP-1 transcription by insulin in diabetes and in rat hepatoma cells, and demonstrated that IGFBP-6 in human cerebrospinal fluid is O-glycosylated. (1) IGFBP-2 mRNA is increased in fasted rat liver (but not brain or kidney), and rapidly normalized by refeeding. Run-on transcription studies demonstrated a corresponding increase in the initiation of transcription of the IGFBP-2 gene. IGFBP-2 transcription and IGFBP-2 mRNA also are increased in neonatal rat liver. (2) Insulin rapidly inhibits IGFBP-1 transcription and IGFBP-1 mRNA in diabetic rat liver. Similarly, in H4-II-E rat hepatoma cells, insulin decreased IGFBP-1 transcription within 20 min. This is a direct effect of insulin, and is mediated by the insulin receptor. The H4-II-E cell line will be used to identify the insulin response elements in the IGFBP-1 promoter. (3) IGFBP-6, one of the predominant IGFBPs in human cerebrospinal fluid, was purified and shown to be O-glycosylated. Enzymatic deglycosylation did not alter its high, preferential affinity for IGF-II versus IGF-I. The effect of O-linked oligosaccharides on the localization of IGFBP-6 in tissues and its biological activity remain to be determined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 51007-12

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin-Cell Interaction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

PI: Ian A. Simpson

Visiting Scientist

DB/NIDDK

Others: Samuel W. Cushman

Chief, EDMNS

DB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Transferred to Z01-DK 48001-01 DB

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 55008-12 MCEB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin's Regulation of Glucose Transport

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Samuel W. Cushman

Chief, EDMNS

DB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES):

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Project has been transferred to Z01-DK 48002 01-DB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK55010-09-MCFB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in Insulin's Action in Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (Last other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Samuel W. Cushman Chief, EDMNS DB/ NIDDK

COOPERATING UNITS (if any)

LAB BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES.

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Project has been transferred to Z01-DK-48003-01 DB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 55012-08

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin's Regulation of Hormone Binding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

PI: Samuel W. Cushman Chief, EDMNS DB/NIDDK

Others: Ian A. Simpson Visiting Scientist DB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Inactive.

Project has been transferred to Z01 DK 48004-01 DB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 55014-07

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in Insulin's Action with Fasting/Refeeding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

PI: Samuel W. Cushman Chief, EDMNS DB/NIDDK
Others: Ian A. Simpson Visiting Scientist DB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been transferred to Z01 DK 48006-01 DB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER ZO1 DK55015-03 MCEB
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT <small>(100 characters or less. Title must fit on one line between the borders.)</small> Mutations of the Thyroid Hormone Receptor Gene in Patients with Thyroid Hormone Resistance		
PRINCIPAL INVESTIGATOR <small>(List other professional personnel below the Principal Investigator.)</small> <small>(Name, title, laboratory, and business affiliation)</small>		
PI: B.D. Weintraub, M.D.	Chief	MCEB, NIDDK
Others: A.J. Mixson	Senior Staff Fellow	MCEB, NIDDK
P. Hauser	Special Volunteer	MCEB, NIDDK
C.A. Meier	Visiting Associate	MCEB, NIDDK
COOPERATING UNITS <small>(if any)</small> Dr. Stephen J. Usala, Section of Endocrinology, East Carolina School of Medicine, Greenville, NC		
LAB/BRANCH Molecular and Cellular Endocrinology Branch		
SECTION Molecular Regulation and Neuroendocrinology Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 3.0	PROFESSIONAL: 3.0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK <small>(Use standard unabbreviated type. Do not exceed the space provided.)</small> <p>Generalized resistance to thyroid hormone (GRTH) is an inherited disease linked to mutations in the beta T3 receptor gene and characterized clinically by the resistance of peripheral and pituitary tissues to the action of thyroid hormone. Of the over 30 different mutations identified in the ligand binding domain of the beta receptor, several were shown to inhibit normal receptor function by a dominant negative mechanism which is likely to mediate the phenotype of this disease. Recent studies have indicated that this dominant negative effect is most likely mediated by competition of mutant and normal receptor for binding to T3 response elements within various thyroid hormone responsive genes.</p> <p>We have also recently investigated whether the heterogeneous phenotypic features that occur within and among kindreds might be due to differential expression of mutant vs. normal receptors in various tissues. Using an allele-specific primer extension method, we have determined that in certain patients there is a great excess of expression of the mutant messenger RNA which may correlate with more severe tissue resistance. The mechanism for such differential expression is currently being examined.</p> <p>We have also investigated the relationship between GRTH and attention deficit hyperactivity disorder. We studied a large population of well characterized patients consisting of 49 affected and 55 unaffected family members comprising a total of 52 adults and 52 children. Attention deficit hyperactivity disorder was found in 70% of affected children compared to 20% of unaffected family members. In addition, thyroid hormone therapy appeared to ameliorate the hyperactivity symptoms in certain patients. Certain of the patients with the hyperactivity disorder were also studied by positron emission tomography. Of the 60 regions measured by positron emission tomography, glucose metabolic rates were significantly decreased in the superior right parietal lobes and increased in the mid-occipital lobes, suggesting that such changes in glucose metabolism may be related to abnormalities of sustained attention.</p> <p>These data demonstrate that an attention deficit hyperactivity disorder is strongly and specifically associated with generalized resistance to thyroid hormone. This is the first defined molecular model of hyperactivity and may provide new insights into the basic pathogenesis of this disorder.</p>		

ANNUAL REPORT

THE LABORATORY OF MOLECULAR AND CELLULAR BIOLOGY

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The LMCB comprises several diverse groups studying a common theme of gene regulation in mammalian cells and their function in the pathophysiology of various disease states. The Molecular Biology Section led by Dr. Carter, studies gene regulation in mammalian cell systems and is particularly interested in developing efficient vector systems for delivery of genes into cells. The Cell Growth and Differentiation Section led by Dr. Oka, is generally interested in the endocrine control of differentiation of the mouse mammary gland and has focused on physiological effects of EGF and the molecular biology of various genes which are important in this process. The Steroid Hormones Section led by Dr. Simons studies the mechanism of regulation of genes via steroid hormones and steroid receptors using techniques of chemistry and molecular biology. Finally a fourth project led by Dr. Tietze and conducted in collaboration with workers in NICHD is aimed at understanding the molecular basis of several human genetic defects which result in lysosomal storage diseases.

Research performed in LMCB has been recognized by various honors, awards and grants to individual members. B. Carter has a substantial three year research grant from the Cystic Fibrosis Foundation for gene therapy of cystic fibrosis. T. Flotte was awarded a Leroy Matthews Physician Scientist Award from the Cystic Fibrosis Foundation to work in the Molecular Biology Section. C. Collier was awarded a PRAT Fellowship to work in the Steroid Hormones Section.

B. Carter was an Invited Symposium Speaker at the 5th Annual North American Cystic Fibrosis Conference (Dallas, TX October 1991) and chaired a Workshop Session on Gene Therapy at the same meeting. B. Carter was also a speaker at the CF Gene Therapy Meeting (November 1991), a Session Chairman at the NIH/NHLBI Meeting on Gene Therapy (Bethesda, MD December 1991), and an Invited Speaker at the NCI Gene Therapy Program Meeting (1992) and the Hemophilia Society Gene Therapy Meeting (1992).

T. Flotte was an Invited Speaker at the Williamsburg Cystic Fibrosis Conference (1992) and the Sixth Annual North American Cystic Fibrosis Conference (October 1992).

S. Simons was an Invited Speaker at the Endocrine Society Annual Meeting June 1991 (Washington, DC) and the 4th International Congress on Hormones in Cancer, September 1991 (Amsterdam, Holland), and chaired a Session on Receptor Biochemistry at the Keystone Symposium on Steroid/Thyroid Receptors (Tamarron, CO, February, 1992).

B. Carter continues to serve fourth terms as an Associate Editor of Virology and on the Editorial Board of Journal of Virology and continues to serve on the WHO International Committee for Nomenclature of Viruses, as Chairman of the NIH Institutional BioSafety Committee and as a member of the NIH Intramural Scientists Procurement Advisory Committee.

S. Simons continues to serve on the Editorial Board of Receptors and has been appointed to the Editorial Board of Journal of Biological Chemistry. S. Simons was also elected President of the NIDDK Assembly of Scientists.

Several members of the laboratory including T. Oka, F. Tietze and S. Simons were invited to write a number of critical reviews in their fields.

Members of the laboratory have maintained international collaborations with groups in Tokyo (Japan), Bet-Dagan (Israel), Heidelberg (Germany), Tel Aviv (Israel) and Basle (Switzerland) as well as collaborations with U.S. laboratories at Rutgers (N.J.), Toledo (Ohio), Ann Arbor (Michigan), U. California (San Francisco) and John's Hopkins Medical School (Baltimore), U. Colorado (Denver).

Function of DNA Virus Genomes in Animal Cells

The group led by B. Carter has continued to employ DNA viruses as molecular probes to study genome expression in human cells. The structure and function of adeno-associated virus (AAV) is being studied intensively. AAV has also been developed as a eukaryotic expression vector.

In a newly developed project, sponsored in part by the Cystic Fibrosis Foundation, we are attempting to develop vectors for possible gene therapy of cystic fibrosis (CF). Thus far the CF defect has been complemented in airway epithelial cells *in vitro* by introduction of an AAV vector containing the cDNA for the CFTR gene. These complementation studies have been achieved both by introducing the AAV vector DNA via cationic liposomes or by infection with AAV transducing particles (i.e. AAV particles containing the vector genome). The latter procedure is remarkably efficient and can provide stable correction of the CF defect in the bulk of the cells in the population. These studies show that the AAV vector is a strong possibility for a gene therapy for CF. Animal studies with AAV vectors are now being initiated prior to proposing human gene therapy trials.

A complex system of gene regulation is mediated by products of the AAV rep gene which are required for replication of AAV DNA and also mediate transcriptional activation and translational inhibition of some genes. Site-directed mutagenesis is being used to resolve the functions of the rep gene. Coding of all these functions in a single gene appears to be unique in eukaryotic systems. A number of very informative mutants of the AAV rep gene were generated. Two alternate expression systems were developed (one using baculovirus vectors in insect cells and one using a transfection system in human cultured cells) which allows expression of mutant AAV rep proteins at high efficiency. This has been used to show that one particular mutant rep protein (which has a DNA-negative, trans-dominant phenotype *in vivo*) is also trans-dominant in vitro. In vitro the mutant rep protein can bind to the AAV replication origin but cannot perform terminal resolution (the first step in AAV DNA replication). Further the mutant rep protein blocks in vitro terminal resolution by the wild type rep protein.

A third project is analysing the interaction between AAV and HIV. Current work suggests that production of vaccines or use of nucleotide analog therapies for AIDS may be limited and difficult. Thus other therapeutic approaches are urgently required. Attempts are underway to develop a novel approach by using the negative regulatory property of a trans-acting gene of the human parvovirus, AAV, to inhibit the function of the trans-acting gene tat, of Human Immunodeficiency Virus (HIV). We have shown in transfection assays that the AAV rep protein can be engineered to be a powerful inhibitor of growth of infectious HIV. We are now analysing the precise mechanism of this effect and attempting to develop delivery systems to introduce Rep into HIV infected cells such that it would be used as an anti-viral therapy for AIDS.

Hormonal Regulation of Cell Growth and Differentiation.

T. Oka's group in the Section of Cell Growth and Differentiation is studying the physiological role and molecular action of growth factors and peptide and steroid hormones such as insulin, glucocorticoid and prolactin on cell growth and differentiation using mammary gland as a model.

A mouse model system for liver regeneration was established and used to show that EGF plays a significant role in the stimulation of liver regeneration. Further studies revealed that EGF receptor gene expression increased rapidly as a result of increased transcription and this increase was blocked by treatment with inhibitors of protein synthesis. In sham operated mice, however, the drug caused a superinduction of the receptor mRNA levels by increasing the stability of the mRNA. These results suggest that transcription induced by partial hepatectomy requires protein synthesis and that labile proteins are involved in the regulation of the stability of EGF receptor mRNA.

The regulatory sequence elements responsible for casein gene expression, are being analysed by constructing casein-CAT chimeric genes and examining their expression in transient assays. These experiments suggest the existence of multiple regulatory elements in the 500 bp 5'-flanking region and corresponding binding factors which are responsible for hormonal induction. One of these DNA binding proteins was found specifically in the mammary gland of pregnant mice, and this protein serves as a repressor that mediates that inhibitory action of progesterone on beta-casein gene transcription. Identification and isolation of other transcription factors for the beta-casein gene is in progress.

A new project has been established to gain further insight into the molecular mechanism of milk protein gene expression. The cDNAs for the two forms of prolactin receptor have been isolated and their expression is being examined in order to gain insight into the molecular mechanism of prolactin action on milk protein gene expression.

Steroid Hormone Action

The action of glucocorticoid hormones is being studied by S. Simons' group in the Steroid Hormones Section. Steroid-induced gene transcription occurs after the receptor-steroid complex binds to specific DNA sequences. A poorly understood step is that of steroid binding to the receptor.

The rate determining step in nuclear binding for glucocorticoid receptors is activation. Various analyses have given rise to the tenet that half saturation of the whole cell receptors by steroid should afford half of the maximal amount of activated complexes, of nuclear bound complexes, and of biological response. Recent results have questioned this model. The objective of the current studies to examine whether the accepted model of steroid hormone action needs to be modified.

Simons' group previously showed that both the concentration of the agonist dexamethasone (Dex) required for half maximal induction (EC_{50}) and the percent agonist activity produced by the partial antagonist dexamethasone 21-mesylate (Dex-Mes) were different for tyrosine aminotransferase (TAT) gene induction in Fu5-5 and HTC rat liver cells. Furthermore, both activities varied over several weeks in each cell line in an apparent random manner. This long term variation can be made to occur reproducibly in ≤ 40 hr simply by changing the cell density and/or amount of medium in the tissue culture plates. A qualitatively identical modulation was seen at the level of TAT mRNA, but not mouse mammary tumor virus RNA. These results further indicate that extra-chromosomal parameters, such as cell-cell contact and/or a diffusible factor(s), can modulate the basic features of glucocorticoid induction of some, but not all, glucocorticoid inducible genes.

No cis-acting sequences, or trans-acting factors having the ability to modulate the activity of steroid receptors had been previously described. Transient transfection assays with hybrid TAT-chloramphenicol acetyltransferase (CAT) genes were conducted in Fu5-5 cells. A 21 bp sequence of the rat TAT gene which acts in concert with a trans-acting factor identified by gel shift experiments was identified. This glucocorticoid modulatory element (GME), located at -3654 to -3634 of the rat TAT gene, is an

authentic transcriptional element in that it conveyed its properties to heterologous genes and promoters and did not synergize with the glucocorticoid response element. The specificity and function of this GME did not correspond to that of any known cis-acting transcriptional elements. These data establish a precedent for the role of steroid modulatory elements in the control of steroid regulated genes.

The current model of steroid hormone action has faithfully guided research in this area for many years. Nevertheless, it now appears that higher level, steroid and receptor independent components (e.g., the GME and its associated factor) must be included to explain how the same receptor-steroid complex can have quantitatively different amounts of activity with associated genes in the same cell.

Lysosomal Transport and Storage Disease

This work is being conducted by Dr. Frank Tietze. Degradation of cellular biopolymers such as proteins and polysaccharides takes place chiefly within the lysosome. The end-products of lysosomal digestion of biopolymers are known to exit the lysosome through the agency of specific membrane carrier proteins. Defects in these carrier proteins can result in a number of inherited storage disorders which are characterized by intralysosomal entrapment of excessive amounts of specific metabolites such as cystine or sialic acid. Unlike the plasma membrane carrier proteins, many of which have been isolated and/or sequenced, nothing is known concerning the structure of the lysosomal membrane carriers.

To further characterize the substrate specificity of the lysosomal membrane sialic acid carrier cultured cells from patients with sialic acid storage disease (Salla disease) were examined to determine if they accumulate abnormal quantities of free L-iduronic acid in addition to sialic acid.

HPLC analysis of protein-free extracts from individuals with sialic acid storage disease did not reveal the presence of free L-iduronic acid in excess of the trace amounts seen in corresponding extracts from normal control cells. In about half of the samples, however, extracts from mutant cells which had been incubated in the presence of heparin showed the presence of elevated amounts of the hexuronic acid.

Sialic, D-glucuronic and L-iduronic acids are three common acidic sugar components of oligosaccharides subject to hydrolysis within fibroblast lysosomes. Previous investigations established that the first two monosaccharides exit the lysosome via the same carrier, the mode of iduronate transport was not established. The inability to detect excess endogenous levels of iduronic acid in the mutant cells may indicate that this sugar utilizes a separate carrier for lysosomal transport or that the defective carrier is "leaky" with respect to this monosaccharide, whose accumulation is only evident after exposure of the mutant cells to an abundant exogenous source of the sugar.

Summary

Research in LMCB overall has followed a common theme of performing basic science on gene expression and also applying this in study of disease states. Thus recent work from this laboratory has identified the basic biochemical defect in one genetic disease (sialuria) illuminated a possible role of EGF deficiency in another disease (diabetes), and is aimed at developing therapeutic approaches to an infectious disease (AIDS) and a genetic disease (cystic fibrosis).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 57501-16

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Function of DNA Virus Genomes in Animal Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Barrie J. Carter	Chief, LMCB	LMCB:NIDDK
Other:	Irving Miller	Biologist	LMCB:NIDDK
	Roland Owens	Senior Staff Fellow	LMCB:NIDDK
	Matthew Weitzman	Visiting Fellow	LMCB:NIDDK
	Terry Flotte	NIH-Johns Hopkins CF Fellow	LMCB:NIDDK
	Rikki Solow	Guest Worker	LMCB:NIDDK
	Sandra Afione	Guest Worker	LMCB:NIDDK
	Sirkka Kystio	Visiting Fellow	LMCB:NIDDK

COOPERATING UNITS (if any)

N. Chejanovsky (Israel), P. Zeitlin, W. Guggino (Johns Hopkins)
D.F. Klessig, B. Antoni, A. Rabson (Rutgers), E. Mendelson (Tel Aviv, Israel)
M. Drumm (Michigan)

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS.

7.0

PROFESSIONAL:

6.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

We are employing DNA viruses as molecular probes to study genome expression in human cells. We are studying intensively the structure and function of a human parvovirus, adeno-associated virus (AAV). AAV has been developed as a eukaryotic expression vector. AAV normally grows in cells only in the presence of a helper virus (either adenovirus or herpesvirus). In the absence of any helper, the AAV genome integrates into the cell chromosome. Thus, the AAV vector is useful as a transducing virus for high frequency integration of genes into mammalian cell chromosomes to yield stable expression. This vector also may be useful for gene therapy. We are now analyzing intensely the control of gene regulation in AAV vectors in order to maximize the expression of foreign genes introduced into mammalian cells using this vector. We are developing AAV vectors that express the CFTR gene as a potential gene therapy for cystic fibrosis. These vectors have been shown to complement the electrophysiological defect in chloride transport in cells from cystic fibrosis patients. We have discovered a complex system of gene regulation mediated by products of the AAV rep gene which are required for replication of AAV DNA but also mediates transcriptional activation and translational inhibition of some genes. Site-specific mutagenesis is being used to resolve these functions. Coding of all these functions in a single gene is unique in eukaryotic systems. Wild type and mutant AAV rep proteins are being expressed in both bacterial insect and mammalian cell expression systems for purification and biochemical analysis. Adenovirus is the helper for AAV. This relationship is being analyzed. Both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus this transformation and also inhibits Ad12 oncogenesis in newborn animals. Thus, AAV inhibits tumor induction. The mechanism of this inhibition of tumor induction is being studied at molecular level cell culture. We also are analyzing interactions of AAV with HIV as potential approach to a novel therapy for AIDS.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57502-19

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hormonal Regulation of Cell Growth and Differentiation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Takami Oka	Senior Investigator	LMCB:NIDDK
Other:	Akio Kanai	Visiting Fellow	LMCB:NIDDK
	Chang-Soo Lee	Visiting Fellow	LMCB:NIDDK
	Robert Moore	IRTA Fellow	LMCB:NIDDK
	Seiji Nishikawa	Guest Worker	LMCB:NIDDK
	Norio Nonomura	Visiting Fellow	LMCB:NIDDK
	John W. Perry	Biologist (Technician)	LMCB:NIDDK

COOPERATING UNITS (if any)

Dr. Laura J. Russell, NICHD, NIH
Dr. Kishio Furuya, National Institute of Physiological Sciences, Japan
Dr. Koichi Enomoto, Shimane Medical University, Japan

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

Cell Growth and Differentiation

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

6.5

PROFESSIONAL:

5.5

OTHER:

1.0

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Beta-casein is the major milk protein synthesized by the mammary gland. The expression of the beta-casein gene undergoes marked changes as a function of reproductive stages. Its expression is low in virgin and postlactating states, whereas the gene is fully expressed in the lactating state. During pregnancy, beta-casein gene expression increases only to a limited extent partly because it is suppressed by progesterone, whose circulating level is increased during this period. Studies using mammary cell culture system have shown that casein gene expression can be induced by prolactin, glucocorticoid, and insulin, but is inhibited by progesterone. Thus, the beta-casein gene in the mammary gland provides a useful model for studying the molecular mechanisms of hormonal control of gene expression during development. We have studies the sequence elements responsible for beta-casein gene expression by transfection of chimeric beta-casein gene into primary mammary epithelial cells. These studies have showed that approximately 500 base pairs of the 5'-flanking sequence of the mouse beta-casein is sufficient for tissue-specific and hormonally induced expression. More recently we examined the sequence-specific binding of mammary nuclear proteins to the promoter region of the beta-casein gene by DNase I footprinting analysis and gel shift assay. These results suggested that multiple cis-acting DNA elements and their binding factors are required for hormonal induction of mouse beta-casein gene expression. In addition, our results revealed the presence of a pregnancy-specific mammary nuclear factor that binds to two separate sites of the beta-casein promoter and indicated that it may serve as a repressor that mediates the inhibitory action of progesterone on beta-casein gene transcription.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57503-19

PERIOD COVERED

October 1, 1992 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Metabolic Defect in Sialuria

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Frank Tietze

Research Chemist

LMCB:NIDDK

Other: None

COOPERATING UNITS (if any)

William A. Gahl, Human Genetics Branch, NICHD

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

-

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It is now known that the monomeric end-products of lysosomal digestion (e.g., amino acids, monosaccharides, etc.) transit the lysosomal membrane to the cytoplasm through the agency of specific porters or carriers, none of which have to date been isolated or characterized. In two instances, inherited defects in lysosomal carrier function are known to result in human disorders with severe clinical consequences viz., cystinosis and sialic acid storage disease. These distinct disorders are characterized, respectively, by abnormal intra-lysosomal accumulations of the amino acid cystine and of the acidic monosaccharides sialic acid and D-glucuronic acid. Since L-iduronic acid, a structural analog of D-glucuronic acid, is a common component of oligosaccharides known to be hydrolytically processed within cellular lysosomes, we have examined cultured skin fibroblasts from patients with sialic acid storage disease for evidence of abnormal accumulation of this hexuronic acid. Such an observed accumulation could be considered presumptive evidence that iduronic and sialic acids share a common lysosomal membrane carrier. HPLC analysis of protein-free extracts of the two variant human forms of sialic acid storage disease (i.e., Salla Disease and infantile sialic acid storage disease) failed to indicate increased endogenous levels of free L-iduronic acid relative to extracts from normal cells, in contrast to the gross elevations noted for sialic acid. However, in two instances mutant cells incubated in the presence of heparin, an oligosaccharide rich in iduronic acid residues, were noted to contain distinctively elevated concentrations of the free acidic monosaccharide. These observations are consistent with the notion that either iduronate utilizes a distant membrane carrier for lysosomal egress or that, if a common porter exists for sialic and L-iduronic acids, the mutant forms characteristic of the sialic acid storage diseases may be "leaky" with respect to the latter sugar, which can accumulate only when the cells are presented with an excess exogenous source of the hexuronic acid.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 57504-05

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Regulation of HIV by AAV

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Barrie J. Carter

Chief, LMCB

LMCB:NIDDK

Other: Roland Owens

Senior Staff Fellow

LMCB:NIDDK

COOPERATING UNITS (if any)

A.S. Rabson and B. Antoni (Rutgers, NJ)

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

-

CHECK APPROPRIATE BOXES

☐ Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The etiologic agent of AIDS is the human immunodeficiency virus (HIV) which differs from most other human viral diseases in exhibiting a very prolonged latent period, but ultimately being lethal due to a profound effect on the immune system. Several trans-acting HIV genes appear to be crucial to HIV growth and infection. Therefore we are studying the feasibility of a novel anti-viral therapy for HIV based on interference by another viral gene with the trans-acting regulation of HIV. The overall goal of this proposal is to analyze interactions between trans-acting regulatory genes of HIV and of a human parvovirus, adeno-associated virus, AAV. We are analyzing the AAV rep gene and its interaction with the HIV tat gene. Current work suggests that developing standard types of anti-viral therapy such as vaccines or nucleotide-analog drugs for HIV is difficult and other alternate possibilities for therapy must be investigated. One approach is to intervene in the trans-regulation system of HIV especially that mediated by the HIV tat gene. Thus a possible anti-viral therapy for HIV is to inhibit the production or the action of tat. A novel way to attempt this is to employ a trans-acting gene from another human virus. One such candidate is the rep gene of the human parvovirus adeno-associated virus (AAV). AAV does not cause any human disease and grows only in cells also infected with adenovirus or herpes viruses. AAV inhibits growth of the helper virus and may play an important role in limiting certain human viral infections. Also AAV can alter important regulatory controls in virus infected cells or in tumor cells. Rep is a novel type of trans-acting regulatory gene which exhibits negative, translational regulation of many genes in several cell types. We are analyzing the AAV rep gene and its interaction with the HIV tat gene. We are testing rep as a potential trans-acting heterologous inhibitor of HIV.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57800-01

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Initial Intracellular Events of Steroid Hormone Action

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	S.S. Simons, Jr.	Chief, Steroid Hormones Section	LMCB:NIDDK
Other:	A.H. Cavanaugh	Staff Fellow	LMCB:NIDDK
	H. Oshima	Visiting Associate	LMCB:NIDDK
	P.K. Chakraborti	Visiting Associate	LMCB:NIDDK
	K.J. Modarress	IRTA Fellow	LMCB:NIDDK
	D. Szapary	Visiting Associate	LMCB:NIDDK
	J. Opoku	IRTA Fellow	LMCB:NIDDK
	C.D. Collier	IRTA Fellow/PRAT Fellow	LMCB:NIDDK

COOPERATING UNITS (If any)

D.P. Edwards (Univ. of Colorado, Denver), W.B. Pratt (Univ. of Michigan Medical School, Ann Arbor), K.R. Yamamoto (UCSF, San Francisco), G. Schütz (German Cancer Research Center, Heidelberg, Germany)

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

Steroid Hormones Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

8.0

PROFESSIONAL:

7.0

OTHER:

-

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The objective of this project is to define the initial, intracellular events of glucocorticoid hormone action and steroid hormone action in general. The first step of steroid binding to the intracellular receptor is followed by activation of the receptor-steroid complex to a DNA/nuclear-binding species that then binds to those nuclear acceptor sites involved in the regulation of transcription of specific genes. It has long been thought both that half saturation of the whole cell receptors by steroid should afford half of the maximal amount of biological response and that the various induction parameters for each agonist and partial antagonist should be invariant. In contrast, our studies of glucocorticoid induction of tyrosine aminotransferase (TAT) revealed that the concentration of a glucocorticoid agonist required for half maximal induction (EC_{50}) and the amount of agonist activity produced by a partial antiglucocorticoid were not the same for all responsive genes within the same cell. Furthermore, both properties for TAT induction varied over a period of several weeks. This variation has now been reproduced in ≤ 40 hr simply by changing the cell density. We are not aware of any previous report of cell growth conditions affecting either the percent agonist activity of a partial antisteroid or the EC_{50} of a full agonist. These variations were found to require the presence of a 21 bp sequence of the TAT gene. We call this 21 bp sequence, which acts in concert with a trans-acting factor identified by gel shift experiments, a glucocorticoid modulatory element (GME). This GME, which is located at -3654 to -3634 of the rat TAT gene, is an authentic transcriptional element to the extent that it conveyed its properties to heterologous genes and promoters. A model incorporating this new element is advanced which can explain, for the first time, the observed variations of TAT induction. Future studies of this model should provide useful information for understanding the control of steroid-regulated gene transcription and the control of gene transcription in general.

1991-2

ANNUAL REPORT OF THE LABORATORY OF ANALYTICAL CHEMISTRY NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The primary mission of the Laboratory of Analytical Chemistry (LAC) now focuses on the provision of analytical services for investigators in NIDDK and elsewhere at NIH and the development of improved analytical methods. Many of the analytical problems brought to LAC for solution involve only application of relatively routine methods, while others are of such complexity that collaborative research efforts are needed.

Included in this report for administrative convenience is the report of the Natural Products Section. This section was abolished at the beginning of FY 1992 upon the retirement of its Chief, Dr. Arnold Brossi, but several projects were completed during the current year.

INSTRUMENTATION SECTION

The instrumental analysis capabilities of LAC are concentrated mainly on nuclear magnetic resonance (NMR), mass spectrometry (MS) and infrared spectroscopy (IR). Several major new instruments were described in last year's report. During FY 1992 no major instruments were acquired, but several upgrades, as discussed below, have augmented the Laboratory's spectral apparatus.

Developments in Mass Spectrometry

During the last two years it has become apparent that normally non-volatile molecules of high molecular weight, such as proteins, can be studied by MS using recent developments in laser desorption and electrospray technology. During this year, the potential mass range for routine samples analyzed within the Laboratory has risen from 1,000 to 50,000 with the introduction of these new techniques and instrumentation. In collaboration with the Laboratory of Biophysical Chemistry, NHLBI, an inter-Institute macromolecular structure determination center is being established. Further information on MS studies and collaborative research problems is included in the annual report of the Laboratory of Bioorganic Chemistry, NIDDK. (Lewis Pannell)

Applications of NMR in Chemical and Biochemical Analysis

During the last year Dr. Yeh has continued to engage in collaborative projects in which he used various NMR techniques to solve complex structure problems. This includes 1) submilligram quantities of epibatidine, a novel chloropyridyl azabicycloheptane with potent analgesic activity from an Ecuadoran poison frog and alkaloid 251F from the dendrobatid poison frog (T. Spande and J. Daly), 2) microbial oxidative metabolites from the action of enzymes in *Beauveria sulfurescens* on the antimalarial drug arteether (Y.L. Hu, H. Ziffer and G.Y. Li), 3) polysaccharides (P. Kovac), 4) colchicoids (O. Boye and A. Brossi), 5) oxohalide complexes of molybdenum and tungsten with oxygen donor ligands (F. Planinic and D. Vikić-Topić), and

6) DNA duplex containing a single centrally positioned covalent adduct between diol epoxides derived from polycyclic aromatic hydrocarbons and the exocyclic amino group of guanosine or adenosine (D.M.Jerina and J.M. Sayer).

Proton NMR is being used to monitor the formation of positional isomers of nucleosides by the action of the enzyme purine nucleoside phosphorylase (PNP), which catalyzes the cleavage and formation of the glycosidic bond of purine nucleosides. The specificity of NMR permits the observation of all possible reaction products which are derived from purine bases and α -D-ribo (or deoxyribo) furanosyl-1-phosphate. Preliminary results showed that human erythrocytic PNP catalyzes the synthesis of not only the commonly known N-9 isomer but also the N-7 isomer of nucleosides as well. For this investigation a number of positional isomers of nucleosides have been prepared and characterized. Detailed mechanistic studies of the formation of isomeric nucleosides by PNP and its possible biological implications are currently underway. (H.J.C. Yeh and G. Y. Li).

NATURAL PRODUCTS SECTION

Research on colchicine to find medically useful tissue-specific spindle toxins has been terminated. Several compounds are presently undergoing a secondary screening. Radiolabeled chloroacetates of 2- and 3-demethylthiocolchicine were found to be highly potent inhibitors of tubulin polymerization, and both covalently reacted with tubulin. It was shown that the ratio of radiolabeled β -tubulin to that of α -tubulin was approximately 4:1 with both compounds.

Research on phenylcarbamate analogs of physostigmine, physovenine and thiaphysovenine has shown that specificity for inhibition of AChE and BChE largely depends on the substitution of the phenyl group of the carbamate moiety. An X-ray analysis of a biologically active and an inactive phenylcarbamate showed that the latter is sterically hindered and makes the carbamoyl group difficultly accessible for a trans-carbamoylation reaction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK58000-47 LAC

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analytical Service and Methodology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E.D. Becker Acting Chief, Lab. Anal. Chem. LAC/NIDDK

Others:

H.J.C. Yeh Research Chemist LAC/NIDDK
N. Whittaker Chemist LAC/NIDDK
W. White Biologist LAC/NIDDK
L.K. Pannell Research Chemist LBC/NIDDK

G.-y Li Visiting Fellow RCB/NCI
H.L. Lee Special Volunteer LAC/NIDDK
Q.-I Pu Special Volunteer LAC/NIDDK
D. Vikić-Topić Visiting Associate LAC/NIDDK
D. Uyakul Visiting Fellow LAC/NIDDK

COOPERATING UNITS (if any)

Laboratories of Bioorganic Chemistry, Medicinal Chemistry, Neuroscience, NIDDK
Radiation Oncology Branch, NCI
Laboratory of Biophysical Chemistry, NHLBI

LAB/BRANCH Laboratory of Analytical Chemistry

SECTION

Instrumentation Section

INSTITUTE AND LOCATION

NIH/NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 7

PROFESSIONAL: 7

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to NIDDK scientists, other NIH laboratories and, to a limited extent, personnel of other government agencies. Instrumental analyses include nuclear magnetic resonance of liquids and solutions; infrared spectroscopy of solids, liquids, and samples introduced by gas chromatography; mass spectrometry (with samples introduced by solids probe, gas and liquid chromatography and capillary zone electrophoresis with ionization by electron impact, chemical ionization, electrospray, thermospray, thermabeam, and fast atom bombardment).

Requests for analyses are obtained from many laboratories and Institutes at NIH, as well as some outside organizations. The laboratory in this way acts as an NIH resource and it is supported, in part, by its collaborators in terms of staffing and equipment where appropriate. Some major instruments are jointly funded and shared with other Institutes to avoid costly duplication of facilities.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-58002LAC

PERIOD COVERED

October 1, 1991 to September 30, 1992.

TITLE OF PROJECT (20 characters or less. Take space from one line between the borders.)

Initial Intracellular Events of Steroid Hormone Action

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator: Name, title, laboratory, and academic affiliation)

COOPERATING UNITS (If any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Project has been transferred to Z01 DK 57800-01

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 58003-19 LAC

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Development of Methods and Materials for the Study of Medical Problems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C.M. Foltz Research Chemist LAC/NIDDK
Others: B. Baer Chemist LAC/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS: 0.2

PROFESSIONAL: 0.1

OTHER: 0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The primary goal of this work is to contribute to the investigation and solution of basic medical problems by the application of chemical, physical and biological methods. This goal is being pursued by studies of the biology and biochemistry of murine tumor cells with emphasis on cancer metastasis.

Studies were begun several years ago to determine whether one or more specific gene products are required to confer on certain tumor cells the properties needed for cancer metastasis. Promising preliminary results obtained with NIH 3T3 cells transfected with one of several oncogenes were reported previously. This work has been suspended temporarily because of other priorities.

Other lines of work in the area of cancer metastasis, such as the interactions of tumor cells with basement membrane components and other biological materials, the nutrition of tumor cells, and the effects of the treatment of tumor cells with chemicals and biologicals on metastatic potencies, are also to be resumed when the priorities imposed by other responsibilities permit. Meanwhile, several lines of murine tumor cells, which have been used in the past and which are to be used in the future, are being maintained in mice and their metastatic potencies monitored.

During this period C. M. Foltz has been serving as NIDDK Safety Officer, Building 8/8A Occupant Emergency Coordinator and Safety Officer, and as Building 8/8A Manager; much of his work has been in those areas. In 1988 Dr. Foltz initiated and developed a course in Laboratory Safety for Summer Guest Workers at NIDDK. With support from the Division of Safety this course is now offered campus-wide. For the past three years there have been 400 to 500 registrants each year.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 58004-25 LAC

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Professional Practices of Biomedical Scientist

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: N. Feder

Medical Officer (Research)

LAC/NIDDK

Others: W.W. Stewart

Research Physicist

LAC/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Analytical Chemistry

SECTION Biophysical Histology

INSTITUTE AND LOCATION

NIH/NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2

PROFESSIONAL: 2

OTHER:

CHECK APPROPRIATE BOX(ES)



(a) Human subjects



(b) Human tissues



(c) Neither



(a1) Minors



(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies have continued on the professional practices of biomedical scientists and on the accuracy of the scientific literature.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 58005-17 LAC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interferon Induction and Action. The Antiviral Activity of Nucleoside Analogs.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator: Name, title, laboratory, and practice affiliation)

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

Project transferred to LMC 59602-19.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-58007-07 LAC

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

PHYSOSTIGMINE AND ANALOGS

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi

Visiting Scientist

LAC/NIDDK

Others: M. Brzostowska
X.S.He

Visiting Scientist

Visiting Scientist

LAC/NIDDK

LAC/NIDDK

COOPERATING UNITS (if any)

Drs. Nigel Greig and S. I. Rapoport, Laboratory of Neuroscience, NIA, NIH; Dr. Q. S. Yu, Institute of Organic Chemistry, Shanghai, China; J. L. Flippen-Anderson, Laboratory of the Structure of Matter, Naval Research Laboratory, Washington, D.C.

LAB/BRANCH: Laboratory of Structural Biology

SECTION: Natural Products Section

INSTITUTE AND LOCATION

NIH/NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 0.3

PROFESSIONAL: 0.3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Several carbamates of the physostigmine and thiaphysostigmine series are presently undergoing a secondary screening to measure half-life and toxicity. Patent applications to cover these compounds as anticholinesterase agents have been filed. An X-ray analysis of a biologically active and inactive representative suggest that the latter is sterically hindered and not capable for trans-carbamoylation of the enzyme.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-58010-06 LAC

PERIOD COVERED

October 1, 1991 to September 30, 1992.

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mammalian Alkaloids.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator (Name, title, laboratory, and institute affiliation))

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Natural Products

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland 20892

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Project has been terminated.

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

STRUCTURE-ACTIVITY RELATIONSHIPS OF COLCHICINOIDS BASED ON TUBULIN BINDING

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi

Visiting Scientist

LAC/NIDDK

Others:

O.Boye

Visiting Fellow

LAC/NIDDK

COOPERATING UNITS (if any)

Dr. E. Hamel, LMP, National Cancer Institute, NIH; Dr. P. Gros, Department of Biochemistry, McGill University, Montreal, Canada; Dr. V. Simanek, Institute of Medicine, Palacky University, Olomouc, Czechoslovakia

LAB/BRANCH: Laboratory of Structural Biology

SECTION Natural Products Section

INSTITUTE AND LOCATION

NIH/NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS 0.3

PROFESSIONAL 0.3

OTHER

CHECK APPROPRIATE BOX(ES)



(a) Human subjects



(b) Human tissues



(c) Neither



(a1) Minors



(a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided)

Radiolabeled chloroacetates of 2-demethyl- and 3-demethyl-thiocolchicine were found to be potent inhibitors of tubulin polymerization. Both compounds formed covalent bonds with tubulin, affording after separation a ratio of 4:1 of radiolabeled beta-tubulin to that of alpha-tubulin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-58014 LAC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analogue of Nucleic Acids and Their Components as Potential Anti-AIDS Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. (Name, title, laboratory, and institute affiliation))

COOPERATING UNITS (if any)

LAB BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unbolded type. Do not exceed the space provided.)

Project has been transferred to LMC 59601-05.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-58017-02 LAC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nortorpane Alkaloids

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES):

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unstructured type. Do not exceed the space provided.)

Project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 58018-02 LAC

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (No characters or less. Title must fit on one line between the borders.)

Analytical Reagents from Dihydroflourescein

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator. (Name, title, laboratory, and agency affiliation)

COOPERATING UNITS (If any)

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL STAFF YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES.

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
 ☐ (a1) Minors
 ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Project has been terminated.

ANNUAL REPORT OF THE LABORATORY OF NEUROSCIENCE

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Studies on the benzodiazepine/GABA receptor chloride channel complex

The benzodiazepine/GABA receptor chloride channel complex ("supramolecular complex") is an oligomeric group of proteins containing recognition sites for many psychopharmacological agents including benzodiazepines, 8-carbolines, and barbiturates. The proteins comprising this supramolecular complex act in concert to regulate the activity of chloride channels that are controlled ("gated") by gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter of the vertebrate central nervous system. Studies are in progress to characterize the molecular aspects of this system, its physiological functions, and possible role in disease.

Recent molecular biological studies indicated the supramolecular complex required at least three distinct but related classes of proteins (termed alpha, beta and gamma) to form a fully functional drug and ligand-gated chloride channel. The establishment of a stable cell line containing cDNAs encoding only two of these classes of proteins (alpha, gamma) that expresses "Type I" benzodiazepine receptors indistinguishable from the native form suggests that the GABA_A receptor complex may also be constituted in heterodimer forms. Nonetheless, neither the number nor arrangement of subunits required to constitute this ligand-gated ion channel are known. Current studies are underway to resolve this issue as well as examine the potential for regulation by (e.g.) drugs in this stably transfected cell line.

The discovery of several high affinity ligands for the "diazepam-insensitive" isoform of benzodiazepine receptor should enable us to design novel agents with selectivity for these sites. These studies should permit us to perform molecular modelling studies of this isoform as well as to elucidate potential pharmacological and physiological functions. This may be of particular significance since Ro 15-4513 (the prototypic ligand for diazepam-insensitive benzodiazepine receptors) can antagonize many of the effects of ethanol in both electrophysiological and biochemical measures and has amethystic properties in vivo.

Previous studies from this laboratory suggest that the GABA_A receptor complex may mediate the pharmacological effects of inhalation agents. The demonstration that an inhalation agent can produce anesthesia in a stereospecific manner in vivo is consistent with a protein rather than lipid target of anesthesia, and permits further study of the locus of action of these agents.

Studies on glycine and glutamate coupled cation channels

We previously demonstrated that functional antagonists at the N-methyl-D-aspartate (NMDA) receptor complex exhibit antidepressant actions in animal models. The high affinity glycine partial agonist, 1-aminocyclopropanecarboxylic acid (ACPC) was as efficacious as the prototypic antidepressant, imipramine, in these

measures. This is of particular importance since ACPC is orally active, has a long duration of action, and does not appear to produce the adverse behavioral effects associated with competitive NMDA antagonists or use-dependent channel blockers. The development of a simple HPLC analytical method to measure this material in biological fluids indicates that both plasma and brain concentrations of this compound are consistent with pharmacological actions in behavioral measures. Moreover, the demonstration that chronic administration of both ACPC and MK-801 produce a downregulation of β -adrenoceptors comparable to imipramine indicates that glutamatergic pathways may be a common pathway of antidepressant drug action. This hypothesis is currently under investigation. The finding that chronic treatment with ACPC results in a desensitization of the NMDA receptor complex led to the demonstration that this regimen effectively reduced the mortality and improved the neurological status of animals subjected to severe forebrain ischemia. The feasibility of applying this regimen to other neuropathologies associated with excessive activation of the NMDA receptor complex is currently under investigation.

The snail toxin Conantokin-G was shown to act as an NMDA antagonist through a specific, noncompetitive inhibition of polyamine responses. However, this peptide appears to act at a previously undescribed locus on the NMDA receptor complex. Studies are currently underway to modify this peptide in order to determine the minimum size and sequence required to produce this action. This and related peptides should be valuable tools in examining the physiological importance of polyamines and their recognition sites in the operation of this ligand-gated cation channel.

Studies on neural-immune interactions

We previously demonstrated that the presence of the LP-BM5 virus mixture in the central nervous system of mice inoculated with this virus mixture as neonates. This virus mixture produces a profound immunosuppression and has been proposed as a murine model of AIDS (MAIDS). The demonstration of cognitive (memory and learning) deficits in these animals using a modified Morris water maze indicates these mice may be a useful non-primate model to study the neuropsychiatric consequences of AIDS. This model may be of particular value since cognitive deficits were evinced prior to the appearance of gross motor or other neurological deficits. Current studies are underway to determine whether drugs that prevent or reduce viral invasion of the central nervous system will affect the cognitive deficits produced by this virus mixture.

Using a simple and reliable method for dual-color analysis of heterogenesis cell populations, it was previously shown that inhibition of calcium influx into splenocytes may be an early event in immunosuppression produced by both physiological (stress) and pharmacological (opiates) means. The finding that pharmacologically relevant concentrations of ethanol and related alcohols effect a similar reduction in mitogen-induced increases in intracellular free calcium is consistent with the notion that this phenomenon may be common to immunosuppression produced by diverse factors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK,58501-06 LN

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Receptors for Neurotransmitters & Drugs in Brain & Peripheral Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute)

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COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Neuroscience

SECTION

Section on Neurobiology

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TOTAL STAFF YEARS:

11

PROFESSIONAL:

11

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unproduced type. Do not exceed the space provided.)

High affinity, stereospecific recognition sites (receptors) for neurotransmitters, neuromodulators, and many clinically useful drugs have been identified in both peripheral tissues and the central nervous system. The interaction of a neurotransmitter, neuromodulator or drug with a specific recognition site initiates a series of events (for example, the opening of an ion channel or activation of an enzyme) resulting in either a physiological response (in the case of a neurotransmitter or neuromodulator) or a pharmacological effect (in the case of a drug). Furthermore, the presence of recognition sites for synthetic substances indicates that endogenous substances may also be present which can mimic (or antagonize) the effects of exogenously applied (synthetic) compounds. Studies are in progress to characterize "recognition-effector" systems with special emphasis on ligand (transmitter)-gated ion channels, to link novel recognition sites to their appropriate effector systems, to isolate and identify novel endogenous ligands of physiological and pathophysiological importance, to develop appropriate model systems to examine these phenomena, and to relate recognition-effector systems to both physiological and pathophysiological processes.

ANNUAL REPORT OF THE MOLECULAR PATHOPHYSIOLOGY BRANCH
National Institute of Diabetes and Digestive and Kidney Diseases

The general goals of the Branch are to investigate normal and abnormal cell function at the molecular level with emphasis on transmembrane signalling by hormones, neurotransmitters, growth factors, and other first messengers acting at the cell surface. Approaches used range from molecular biologic techniques to clinical investigation in an effort to define the pathogenesis of diseases characterized by abnormal signal transduction.

Guanine nucleotide binding proteins (G-proteins) as receptor-effector couplers

A family of G-proteins functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for polypeptide hormones, monoamine neurotransmitters, photons of light, chemical odorants, chemotactic factors, and certain growth factors. Effector functions regulated by G-proteins include cAMP formation, cGMP degradation, phosphoinositide breakdown, and several types of ion channel. Major areas of interest concerning G-proteins include: 1) definition of the diversity within this gene family; tissue and subcellular distribution; regulation of gene expression. 2) definition of domains on individual G-protein subunits involved in association of the subunits, attachment to cell membranes, interaction with receptor and effector domains, and possible interactions with other regulatory proteins. 3) definition of the degree and mechanism of specificity for individual G-proteins in coupling to both receptors and effectors. 4) definition of quantitative and qualitative alterations in G-proteins that result in altered signal transduction. Significant recent progress has been made in each of these areas:

1) Molecular basis for subunit association and membrane attachment of GTP-binding proteins- G-proteins are heterotrimers; alpha subunits reversibly associate with a beta/gamma complex. The holoprotein is associated with the cytoplasmic side of the plasma membrane, but the basis for membrane attachment and the domains responsible for subunit association have not been defined. By metabolic labeling of transfected cells and immunoprecipitation of expressed alpha subunits, we can show that Gi but not Gs-alpha subunits undergo a specific fatty acid acylation (myristoylation). Site-directed mutagenesis of the myristoylation site has been performed. The mutant alpha subunit fails to be myristoylated, and upon transfection in cos cells, remains in the cytosol, unlike the wild-type which is targeted to the cell membrane. This highlights the critical role of this covalent modification in membrane attachment. The myristoylation defective Gi alpha subunit has now been expressed in sf9 cells using a baculovirus vector. The mutant alpha subunit, as predicted, was expressed solely in the soluble fraction, and comprised a substantial fraction (dominant band on Coomassie blue-stained gels) of total cell protein. Its solubility permitted facile purification with a novel approach using ammonium sulfate precipitation and dye-affinity chromatography. The purified protein specifically bound guanine nucleotides, served as a pertussis

toxin substrate, and interacted with beta/gamma (with lower affinity as predicted from its lack of myristoylation). Availability of mg quantities of pure protein should facilitate structural and functional studies.

A "CAAX" motif at the carboxy terminus of certain proteins, including the G protein gamma subunits, undergoes complex post-translational processing critical for membrane targeting. A superficially similar motif (CGLF or CGLY) is found at the carboxy-terminus of the pertussis-sensitive G protein alpha subunits, and we have shown previously that this does not undergo processing, presumptively because of the glycine residue. Mutation of the Gil-alpha C-terminus to CVLS or CALL is now shown to lead to processing with isoprenylation with farnesyl (C15) or geranylgeranyl (C20) respectively. This emphasizes the role of the last residue in defining specificity of isoprenylation. Interestingly, despite the increased hydrophobicity associated with the isoprenylation, neither form becomes membrane-associated when the C-terminal mutation is added to the myristoylation-defective alpha subunit. This points out that lipid modification alone is insufficient for membrane targeting [with P. Backlund, NIMH].

The basis for Gs-alpha attachment remains unclear. Extensive mutagenesis studies including amino and carboxy terminal as well as double deletions indicate that neither the extreme amino- nor carboxy-termini are critical for Gs-alpha localization to the particulate fraction of transfected cos cells. This may reflect the role of multiple domains in Gs-alpha membrane attachment. Further mutagenesis and biochemical studies are underway. [Drs. Jones, Juhn, Detgarjev, and Spiegel, in collaboration with Dr. Backlund]

2) Studies of the structure and function of the beta/gamma dimer:

a) Characterization of gamma subunit expression and postranslational processing- Previous work has established the importance of a C-terminal "C-A-A-X" domain present in gamma subunits to signal a complex trio of postranslational modifications: isoprenylation of the cysteine sulfur, truncation of the C-terminal three amino acids, and carboxylmethylation of the free α -carboxylic acid. Mutation of the C-A-A-X cysteine to serine in γ_2 permitted association and coexpression with the β subunit, but blocked membrane attachment of the $\beta\gamma$ complex. Analysis of the [^3H]isoprenoids released by methyl-iodide cleavage from recombinant γ_1 , γ_2 and γ_3 subunits expressed in [^3H]mevalonic acid labeled COS cells reveals comigration on reverse phase HPLC with C15 farnesyl (γ_1) and C20 geranylgeranyl (γ_2 and γ_3). This correlates with the relatively soluble distribution of $\beta\gamma_1$ compared to $\beta\gamma_2$ and $\beta\gamma_3$ in hypotonic cell lysates and parallels the behavior of $\beta\gamma_1$ in hypotonic extracts of retinal rod outer segment membranes. This confirms the ability of COS cells to differentially isoprenylate γ subunits and suggests the variable processing may govern cellular distribution. [Drs. Simonds and Spiegel, in collaboration with Dr. Backlund]

b) Functional effects of gamma mutation on $\beta\gamma$ targeting and function in stable transfectants- Because the nonisoprenylated γ_2 mutant assembles with the β subunit but distributes to the cytosol, we hypothesized that this would impart a dominant inhibitory phenotype in mutant-bearing cells since both receptor and $G\alpha$ are located in the membrane. C6 glioma cells were stably transfected with the nonisoprenylated γ_2 mutant and immunoblot analysis revealed that a portion of endogenous β subunits were shifted to the cytosol, unlike cells transfected with wild-type γ or vector alone. This demonstrated that the exogenous mutant γ was capable of assembling with and diverting at least a portion of the endogenous β pool to the cytosol. Preliminary functional studies of β -adrenergic stimulated cAMP formation in stable transfectants reveal a blunted response to agonist in mutant γ -bearing but not control cells. [Drs. Simonds and Spiegel, with Dr. Collins]

c) Effector modulation by the $\beta\gamma$ complex: adenylylcyclase stimulation- The cloning and expression of multiple adenylylcyclase subtypes has revealed a subtype-specific modulation by $\beta\gamma$. The endogenous adenylylcyclase in COS cells exhibits a stimulatory response to $\beta\gamma$ conditional on the presence of activated $G\alpha$. Transient transfection of COS cells with β and γ cDNAs and subsequent cholera toxin treatment increases cAMP accumulation relative to vector-transfected toxin treated cells. Preliminary results suggest that the nonisoprenylated γ_2 mutant fails to demonstrate this effect. [Dr. Simonds]

d) Mutagenesis of the β subunit. The amino terminus of the β subunit is predicted by computer algorithms to form an α -helix and to favor formation of a coiled-coil interaction. Unlike wild-type β , which requires cotransfection with γ for expression, an amino-terminal β deletant which lacks the coiled-coil region is well expressed in the particulate cell fraction with or without γ . This deletant furthermore fails to shift to the cytosol when coexpressed with the nonisoprenylated γ_2 mutant, unlike the wild type β . Whereas wild-type squid β fails to coexpress with mammalian γ_2 , a chimeric molecule containing ~10% of the mammalian β sequence comprising its coiled-coil domain and ~90% squid β sequence demonstrated γ_2 interaction. These results led to the recognition of a coiled-coil domain in the γ subunit and generation of a molecular model of $\beta\gamma$ interaction based on a coiled-coil interaction in which a core hydrophobic interaction is reinforced by flanking ionic bonds between the two α -helices. [Drs. Garritsen and Simonds, in collaboration with Dr. Van Galen]

3) Altered G-proteins as a cause of altered signal transduction- As critical intermediates in the signal transduction pathway, quantitative or qualitative alterations in G-proteins could have a major impact on the signalling process. We have generated mutant alpha subunits for several G proteins that should lead to either constitutive activation or dominant inhibition of G protein function. The mutant alpha subunits have been initially characterized after transient expression in cos

cells. Mutant Gi2-alpha cDNAs have been stably transfected into NIH 3T3 cells. The constitutively active mutant causes increased cell proliferation, while the dominant inhibitory mutant slows growth. Comparable mutants of Gi1 also stimulate cell proliferation when stably transfected in NIH3T3 cells but those of Gi3-alpha do not. In the latter case, immunolocalization studies indicate that Gi3 may be preferentially targeted to the golgi rather than plasma membrane. This raises interesting questions regarding differential distribution and function of specific G protein subtypes. [Drs. Hermouet, Merendino, and Spiegel]

4) Specificity of G protein receptor-effector coupling- We have made specific peptide antibodies against G protein alpha subunits. Antisera raised against the carboxy-termini of alpha subunits are capable of uncoupling receptor from G protein in native membranes, and can be used to define the specificity of coupling. In the insulin-secreting RIN cell line, a Gi1/Gi2-alpha-specific antibody selectively blocks coupling to the galanin receptor that mediates inhibition of insulin secretion [with G. Sharp, Cornell]. Studies in Hela cells transfected with the serotonin 5HT1A receptor subtype show that an antibody against the Gi3-alpha protein uncouples the transfected receptor from both pertussis toxin sensitive effector pathways in these cells (inhibition of adenylyl cyclase and stimulation of phospholipase C). This provides evidence that a single G protein can couple to dual effectors [with R. Lefkowitz, Duke]. The Gs-alpha specific antibody was used to show that this protein interacts directly with the skeletal muscle calcium channel [with L. Birnbaumer, Baylor], and that TSH stimulates proliferation of a cultured rat thyroid cell line through the Gs protein [with J. Feramisco, UCSB]. In collaboration with the latter group, affinity purified antibodies have been microinjected into living cells to permit direct demonstration of the role of specific G protein alpha subunits in key signal transduction responses. The Gi2-specific antibody, for example, blocked mitogenesis in response to serum factors in swiss 3T3 cells, whereas the Gq antibody (specific for a novel class of pertussis toxin-insensitive G proteins linked to phospholipase C stimulation) blocks increases in intracellular calcium and mitogenesis in response to bradykinin. [Drs. Shenker, Goldsmith, and Spiegel, in collaboration with Dr. Unson]

Pseudohypoparathyroidism (PHP)

PHP is a genetic disorder in which resistance to parathyroid hormone (PTH) may be associated with somatic abnormalities collectively termed Albright's hereditary osteodystrophy (AHO). We have previously shown that subjects with this form of PHP are resistant to multiple hormones that act by stimulating cAMP formation, that an approximate 50% reduction in activity of the G-protein (Gs) that couples receptors to stimulation of adenylyl cyclase is present in all tissues from affected subjects, and that subjects with PHP show reduction in steady state mRNA for the Gs-alpha subunit. We have now succeeded in defining the genetic abnormality responsible for Gs deficiency. Using the polymerase chain reaction to amplify genomic fragments encompassing each exon of the Gs-alpha gene, and comparing such fragments from

normal and affected subjects on denaturing gradient gel electrophoresis, we were able to identify fragments with abnormal mobility. By direct DNA sequencing such fragments contained mutations that would explain reduction in mRNA in affected subjects. This work indicates that mutations in a G protein gene can lead to clinically significant disease. [Drs. Weinstein, Friedman and Spiegel, in collaboration with Dr. Gejman]

McCune-Albright syndrome (MAS)

MAS is a non-genetic disorder in which affected subjects show a variety of seemingly unrelated abnormalities including polyostotic fibrous dysplasia, pigmented skin lesions (cafe-au-lait spots), and autonomous hyperfunction of various endocrine organs including gonads, anterior pituitary, thyroid, and adrenal cortex. The endocrine abnormalities lead to precocious puberty, gigantism, hyperthyroidism, and hypercortisolism. The cause of this sporadic disorder has been completely enigmatic, but speculations have centered on a defect in signal transduction leading to endocrine hyperfunction. The distribution of skin lesions has also suggested the possibility of a somatic mutation acquired early in embryogenesis and affecting only a subset of cells (mosaicism). Since a G protein mutation could plausibly explain the endocrine manifestations, we searched for and found mutations of the Gs-alpha gene that lead to constitutive activation of the Gs protein. These mutations were found in a mosaic distribution; notably, mutant gene was undetectable in normal-appearing portions of endocrine glands, but was present at heterozygous levels in neoplastic portions of endocrine tissue. Mutant Gs-alpha was also detected in dysplastic bone lesions. Occurrence of mutant Gs-alpha in organs such as heart and liver suggest a possible role in "non-classical" manifestations, including sudden death. Our studies suggest that MAS is caused by a somatic mutation in the Gs-alpha gene occurring early in development and found in a mosaic distribution. [Drs. Weinstein, Shenker and Spiegel, in collaboration with Dr. Gejman]

Nephrogenic diabetes insipidus (NDI)

NDI is an inherited X-linked disorder in which affected subjects are resistant to the actions of vasopressin (AVP) on renal medullary cells responsible for water concentration. Clinical manifestations include severe polydipsia and polyuria, and resultant severe dehydration can lead to cerebral swelling and death. Treatment with a potent AVP analog (DDAVP), useful in other forms of DI, is ineffective in NDI because of end-organ resistance to the hormone. The renal actions of AVP are mediated through a V2 type receptor linked via the Gs protein to stimulation of the 2nd messenger cAMP. In theory, the inherited gene defect could be located anywhere along the signal transduction path, but indirect evidence suggested a likely receptor defect. The recent cloning of a human V2 receptor permitted chromosomal localization studies which showed that the receptor is localized to Xq28, the site of the gene defect as determined by family linkage studies. This strongly suggested but did not prove that a receptor gene mutation is the underlying defect in NDI. We have obtained genomic DNA

samples on multiple families with NDI, and in one family thus far have identified a mutation predicted to disrupt formation of a normal V2 receptor. These findings have important implications for our understanding of the pathogenesis of NDI and of normal V2 receptor structure and function, for identification of affected subjects and carriers, and eventually for gene therapy of the disease. [Drs. Merendino and Spiegel, in collaboration with Drs. Lolait and Brownstein, NIMH].

Molecular biologic studies on the cause of parathyroid neoplasia

Parathyroid tumors (benign adenomas, hyperplasia, and carcinoma) are presumptively due to acquired (and in some cases such as multiple endocrine neoplasia type I {MEN I} to inherited) abnormalities at the gene level. We are studying the molecular basis for parathyroid neoplasia by searching for mutations, rearrangements and/or deletions in genomic DNA from parathyroid tumors. We have found rearrangement of the parathyroid hormone gene in only 1 of 43 parathyroid adenomas, but this gene abnormality may be pathogenetically relevant. In contrast, point mutations in ras oncogenes were not found in any parathyroid tumors. In both "hyperplastic" glands from subjects with MEN I and in sporadic adenomas loss of heterozygosity for loci on chromosome 11q13 was found. The data show that tumors in MEN I are monoclonal, and that a locus on 11q13 may encode a tumor "suppressor" gene. By mapping deletions at 11q13 in parathyroid tumors, we are attempting to identify the MEN I gene.

Honors and Awards:

Lee Weinstein (Senior Staff Fellow) gave a lecture at the Annual Meeting of the Canadian Society for Clinical Chemists in Toronto, and also was invited to lecture and to serve on the scientific committee at the International Sero Symposium: "Endocrinology under 35," in Italy. Dr. Weinstein's abstract on the McCune-Albright syndrome was selected for oral presentation at a plenary session of the Annual Meeting of the American Society for Bone and Mineral Research.

Andrew Shenker (Senior Staff Fellow) gave an oral presentation at a plenary session of the Annual Meeting of the Society for Pediatric Research.

Allen Spiegel (Branch Chief) was invited to lecture at National and International Meetings including the 11th Joint Meeting of the British Endocrine Societies, the Annual Meetings of the American Gastroenterology Association and the American Diabetes Association, the 8th International Conference on 2nd messengers, and the Sero Symposium on G protein-coupled receptors.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 59000-5 MPB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular biologic studies on the cause of parathyroid neoplasia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D.

Chief, Molecular Pathophysiology Branch, NIDDK

Others: E. Friedman, M.D.,

Senior Staff Fellow, MPB, NIDDK

COOPERATING UNITS (if any)

S. Marx, M.D., Chief, Mineral Metabolism Section, MDB, NIDDK

LAB/BRANCH

Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Primary hyperparathyroidism (HPT), a common endocrine disorder that can cause significant morbidity, may be due to benign neoplasia of a single parathyroid gland (adenoma) or multiple parathyroid glands (hyperplasia), and rarely, to malignant neoplasia of a parathyroid gland (carcinoma). The etiology of parathyroid neoplasia has not been defined. As with other forms of neoplasia, parathyroid tumors are presumably due to inherited (germ-line mutation) and/or acquired (somatic mutation) defects in specific genes. Etiologic genetic defects could include inappropriate expression of transforming "oncogenes" and/or loss of expression of tumor "suppressor" genes. The availability of surgically resected parathyroid tumors from patients with sporadic and hereditary forms of disease allows us to search for tumor-specific genetic abnormalities that may be involved in development of parathyroid neoplasia.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 59001-27 MPB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Guanine nucleotide binding proteins as receptor-effector couplers

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D., Chief, MPB, NIDDK

Others: T. Jones, M.D., Senior Clinical Inv., MPB, NIDDK

P. Goldsmith, Ph.D., Res. Biol., MPB, NIDDK; M. Degtjarev, Visit. Fell,

Y.-S. Juhn, M.D. Visiting Fellow, MPB, NIDDK MPB, NIDDK

J. Merendino, M.D., Med. Staff Fellow, MPB, NIDDK

S. Hermouet, M.D., Visiting Fellow, MPB, NIDDK

N. Thambi, Ph.D. Visiting Fellow, MPB, NIDDK

A. Shenker, M.D., Senior Res. Inv., MPB, NIDDK

COOPERATING UNITS (if any)

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P. Backlund, NIMH

J. Feranisco, UCSD, La Jolla CA.

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Molecular Pathophysiology Branch

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NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

10

PROFESSIONAL:

8

OTHER:

2

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☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

A family of guanine nucleotide binding proteins (G-proteins) functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for hormones, neurotransmitters, light, odorants, and certain growth factors. Effector functions regulated (positively and, in some instances, negatively) by G-proteins include cAMP formation, phosphoinositide breakdown, potassium and calcium channels, and cGMP degradation. We have used a variety of techniques to study the expression, distribution, regulation, structure and function of G-proteins. Our studies highlight the diversity within the G-protein family. Using peptide specific antibodies, we have defined the specificity of G-proteins in coupling to receptors and effectors. We have defined distinct post-translational lipid modifications necessary for membrane attachment of G protein alpha and beta/gamma subunits. We have created mutations in alpha subunits that cause constitutive activation, and transfected these into cells to define phenotypic effects on cellular function. These studies provide the basis for understanding the role of G-proteins in normal signal transduction and for elucidating possible defects in G-protein structure or function as the basis for abnormal signal transduction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01-DK 59002-27 MPB
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) <u>Studies on pseudohypoparathyroidism and related disorders</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) PI: L. Weinstein, M.D. Senior Staff Fellow, MPB, NIDDK Others: A. Spiegel, M.D. Chief, MPB, NIDDK S.-H. Yu, Ph.D. Visiting Associate, MPB, NIDDK P. DeMazancourt, Ph.D. Visiting Fellow, MPB, NIDDK		
COOPERATING UNITS (if any) P. Gejman, Clin. Neurogenetics, NIMH		
LAB/BRANCH Molecular Pathophysiology Branch		
SECTION		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS 1.0	PROFESSIONAL: 0.5	OTHER 0.5
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreserved type. Do not exceed the space provided.) <p>In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism" (PHP). Patients with this disorder show characteristic constitutional features (Albright's hereditary osteodystrophy - AHO) and do not respond to exogenous parathyroid hormone (PTH). In PHP, UcAMP (urinary cyclic AMP) does not increase normally in response to PTH administration. This indicates that there is a defective hormone <u>receptor-adenylate cyclase complex</u> in this disorder. We have shown that many patients with PHP+AHO (PHP Ia) show an approximately 50% reduction in activity of Gs (the stimulatory guanine nucleotide binding protein associated with adenylate cyclase) in membranes from multiple tissues. Gs deficiency presumably accounts for resistance to multiple hormones in such patients. Using cloned human cDNA probes for the alpha subunit of Gs, we have shown that steady state mRNA levels from fibroblasts of subjects with PHP Ia are reduced by approximately 50% compared with normals. We have now succeeded in defining the genetic abnormality responsible for Gs deficiency. Using the polymerase chain reaction to amplify genomic fragments encompassing each exon of the Gs-alpha gene, and comparing such fragments from normal and affected subjects on denaturing gradient gel electrophoresis, we were able to identify fragments with abnormal mobility. By direct DNA sequencing such fragments contained mutations that would explain reduction in mRNA in affected subjects.</p>		

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 59003-02 MPB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on McCune-Albright Syndrome

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D., Chief, MPB, NIDDK

Others: L. Weinstein, M.D., Senior Staff Fellow, MPB, NIDDK

A. Shenker, M.D., Ph.D., Senior Research Inv., MPB, NIDDK

COOPERATING UNITS (if any)

P. Gejman, Clin. Neurogenetics Branch, NIMH

LAB/BRANCH

Molecular Pathophysiology Branch

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TOTAL MAN-YEARS

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☒ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

McCune-Albright syndrome (MAS) is a non-inherited disorder in which affected subjects show a variety of seemingly unrelated abnormalities including the classic triad of polyostotic fibrous dysplasia, pigmented skin lesions (cafe-au-lait spots), and autonomous hyperfunction of various endocrine organs including gonads, anterior pituitary, thyroid, and adrenal cortex. The endocrine abnormalities lead to precocious puberty, gigantism, hyperthyroidism, and hypercortisolism. The cause of this sporadic disorder has been completely enigmatic, but speculations have centered on a defect in signal transduction leading to endocrine hyperfunction. The distribution of skin lesions has also suggested the possibility of a somatic mutation acquired early in embryogenesis and affecting only a subset of cells (mosaicism). Since a G protein mutation could plausibly explain the endocrine manifestations, we searched for and found mutations of the Gs-alpha gene that lead to constitutive activation of the Gs protein. These mutations were found in a mosaic distribution; notably, mutant gene was undetectable in normal-appearing portions of endocrine glands, but was present at heterozygous levels in neoplastic portions of endocrine tissue. Mutant Gs-alpha was also detected in dysplastic bone lesions. Occurrence of mutant Gs-alpha in organs such as heart and liver suggest a possible role in "non-classical" manifestations, including sudden death. Our studies suggest that MAS is caused by a somatic mutation in the Gs-alpha gene occurring early in development and found in a mosaic distribution.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 59004-01 MPB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Guanine nucleotide binding protein beta-gamma dimers: structure and function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

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Senior Clinical Investigator, MPB, NIDDK

Others:

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A. Garritsen, Ph.D.

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T. Murakami, M.D.

Visiting Associate, MPB, NIDDK

C. Lee, Ph.D.

Visiting Associate, MPB, NIDDK

H. Manji, M.D.

Senior Staff Fellow, ETB, NIMH (Part-time)

COOPERATING UNITS (if any)

C. Unson, Rockefeller University

P.J.M. Van Galen, LBC, NIDDK

P. Backlund, NIMH

R. Collins, MPB, NIDDK

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TOTAL MAN-YEARS

2.0

PROFESSIONAL:

2.0

OTHER:

0

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☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The guanine-nucleotide binding regulatory proteins (G-proteins) are $\alpha\beta\gamma$ heterotrimers which function as transmembrane signal transducers by coupling receptors for extracellular stimuli to intracellular effectors (enzymes, ion channels). G-proteins constitute a diverse family distinguished by specific receptor and effector interactions which in turn are determined by the structure of the three constituent subunits. The α subunit binds guanine nucleotides and has a well established role in effector modulation. The β and γ subunits are tightly associated as a $\beta\gamma$ complex, comprising a single functional entity which, like the α subunit, is absolutely required for G-protein interaction with receptor. An effector modulatory role for the $\beta\gamma$ complex is becoming increasingly apparent in several systems. The present research emphasizes the role of the $\beta\gamma$ complex in G-protein-mediated signal transduction. We have used subunit specific peptide antibodies to probe regions of the $\beta\gamma$ complex important for functional interaction with the α subunit and to monitor expression of recombinant subunits. Site-directed mutagenesis has been used to study the assembly, processing and effector function of the $\beta\gamma$ complex in both transient and stable transfected cell systems. These studies may elucidate the contribution of the $\beta\gamma$ subunit complex to the receptor and effector selectivity characteristic of G-proteins and to the adaptive responses pursuant to agonist stimulation.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 59005-01 MPB

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on nephrogenic diabetes insipidus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D. Chief, MPB, NIDDK
Others: J. Merendino, M.D. Clinical Associate, MPB, NIDDK (MSF)
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Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Nephrogenic diabetes insipidus (NDI) is an inherited X-linked disorder in which affected subjects are resistant to the actions of vasopressin (AVP) on renal medullary cells responsible for water concentration. Clinical manifestations include severe polydipsia and polyuria, and resultant severe dehydration can lead to cerebral swelling and death. Treatment with a potent AVP analog (DDAVP), useful in other forms of DI, is ineffective in NDI because of end-organ resistance to the hormone. The renal actions of AVP are mediated through a V2 type receptor linked via the Gs protein to stimulation of the 2nd messenger cAMP. In theory, the inherited gene defect could be located anywhere along the signal transduction path, but indirect evidence suggested a likely receptor defect. The recent cloning of a human V2 receptor permitted chromosomal localization studies which showed that the receptor is localized to Xq28, the site of the gene defect as determined by family linkage studies. This strongly suggested but did not prove that a receptor gene mutation is the underlying defect in NDI. We have obtained genomic DNA samples on multiple families with NDI, and in one family thus far have identified a mutation predicted to disrupt formation of a normal V2 receptor. These findings have important implications for our understanding of the pathogenesis of NDI and of normal V2 receptor structure and function, for identification of affected subjects and carriers, and eventually for gene therapy of the disease.

ANNUAL REPORT OF THE LABORATORY OF MEDICINAL CHEMISTRY NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The major research direction of the Laboratory is the elucidation of the structure and function of neurotransmitter systems in the mammalian central nervous system (CNS) and the molecular mechanism of action of CNS active drugs. Also under investigation are the detailed interaction, on a molecular level, between antibodies and antigens, peripheral signaling systems and the mechanisms through which the immune and other peripheral systems are influenced by the CNS in normal and disease states, and the exploration of novel approaches to drugs to control cellular proliferation and virus growth. Organic/medicinal chemistry is the foundation of the multidisciplinary approach utilized in these studies which requires synthesis of novel agonists, antagonists, imaging agents, affinity ligands and other drugs for particular applications.

Drug Design and Synthesis Section

Present work in the Drug Design and Synthesis Section of this Laboratory is concerned with rational design and the synthesis of new, highly selective ligands for drug receptors, using all of the contemporary tools of medicinal chemistry, including computer assisted molecular modeling. Areas now under intense investigation include: (1) central opioid receptor subtypes and peripheral opioid receptors, (2) binding sites on components of the immune system which resemble central opioid receptor subtypes, (3) the mechanism of cocaine and narcotic tolerance and dependence (4) phencyclidine (PCP) recognition sites, (5) sigma, cannabinoid (marijuana) and central and peripheral benzodiazepine receptors and (6) development of new ligands for positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging of drug receptors in the CNS of living animals and conscious humans. The multidisciplinary nature of this program requires extensive collaboration with other groups from within and outside of NIH for the purpose of discernment of the structure and function of these receptors and to ensure the practical utility of the discovered ligands provided to biological and biochemical researchers. This Section is involved in collaborative work with, among others, researchers at the University of Alabama, the University of Arizona, the University of Michigan Medical School, the Medical College of Virginia, Meijo University in Japan, The University of Illinois Medical School in Peoria, the Naval Research Laboratory, the Walter Reed Army Institute of Research, the National Institute of Mental Health and the National Institute on Drug Abuse (ADAMHA), the Nuclear Medicine Department of the Warren Grant Magnuson Clinical Center of NIH, the National Heart, Lung and Blood Institute of NIH, the National Institute of Neurological Disorders and Stroke of NIH, G. D. Searle and Co., Neurogen Corp., and the Laboratory of Neuroscience of NIDDK.

Behavioral Pharmacology Unit of the Drug Design and Synthesis Section

Assesses pharmacological agents designed to modify the behavioral effects of drugs of abuse. Other capabilities have been developed to explore pharmacological properties of CNS-active drugs, in an effort to have access to rodent models to screen effects of agent before testing in primates. The neuroendocrine and immune function in plasma of drug-trained monkeys will be examined in the future, and PET scanning will be utilized before and after exposure to drugs of abuse in order to determine potential changes in various neurotransmitter systems as a consequence of prior drug exposure. These studies are unique in that they will investigate potential differences in animals working for drug, as opposed to simple non-contingent exposure to drug. Previous studies have linked susceptibility to drug abuse with individual differences in susceptibility to stress activation of the hypothalamic pituitary adrenal (HPA) axis. The primary endogenous agents involved in the HPA axis are corticotropin releasing hormone (CRH), adrenocorticotrophic hormone and cortisol. Several studies have been directed at examining the behavioral specificity of the direct effects of CRH on behavior.

Biomedical Chemistry Section

In the Biomedical Chemistry Section, LMC, interferon-induced enzymes, especially those dependent on nucleic acids for action, are studied in order to understand them as nucleic acid receptor systems as well as to elucidate their fundamental cellular role. In addition, this research seeks to attempt to explore the application of the 2-5A system as an approach to the chemotherapy of viral or neoplastic disease. New nucleoside analogues are prepared and evaluated for their antiviral/antitumor

potential. This Section is involved in collaborative research with researchers in the Rega Institute in Belgium and at the USUHS.

Section on Carbohydrates

The Section on Carbohydrates (SOC), LMC, works on the interaction of (complex) carbohydrate determinants with monoclonal antibodies (MAbs). The elucidation of this interaction - in great molecular detail - is important since it pertains to all ligand-protein interactions. Thus, drug-receptor, effector-receptor as well as viral-receptor interactions may be clarified. We are executing:

1. Physico-chemical studies on antibody/antigen systems.
2. The synthesis of ligands for affinity studies.
3. The manipulation of immunoglobulin genes to produce specifically mutated genes expressing altered antibodies.
4. The study of immunodeterminants of bacteria causing significant diseases on a global scale, so as to evaluate procedures for vaccine development.

The SOC has determined the specific interaction between microbial polysaccharides such as dextran and a number of monoclonal antibodies in the past. The SOC has prepared many complex fragments of the capsular polysaccharide of *Shigella dysenteriae* type 1 by sophisticated syntheses, and have mapped the binding area of a monoclonal antibody towards this disease-causing micro-organism. In this manner, the immuno-determinant of this polysaccharide could be defined. Both the variable region of the heavy (VH) and the light (VL) chains have been cloned and sequenced, and they have been incorporated in a bacterial expression vector. The VH and VL are linked by a short DNA sequence coding for a fifteen amino acid peptide so that the expressed protein is a covalently linked FV. Expression is presently going on in *E. Coli*. This Section is involved in collaborative research with scientists at Columbia University, in NICHD, NIH, and in Czechoslovakia.

The following summary describes selected advances made by the three Sections in the Laboratory of Medicinal Chemistry during 1991-1992.

DRUG DESIGN AND SYNTHESIS SECTION

Opioid Receptors

Epimeric 6 α - and 6 β -iodo-3,14-dihydroxy-17-cyclopropylmethyl-4,5 α -epoxymorphinans as Potential Ligands for Opioid Receptor Single Photon Emission Computed Tomography (SPECT): Synthesis, Evaluation and Radiochemistry of [125]6 β -iodo-3,14-dihydroxy-17-cyclopropylmethyl-4,5 α -epoxymorphinan ([125]ioxy) - The epimeric 6 β - and 6 α -iodo-3,14-dihydroxy-17-cyclopropylmethyl-4,5 α -epoxymorphinans (1, ioxy) and (2, epioxy), respectively, were each synthesized in 5-steps starting with naltrexone. The configuration of the 6-iodo group of 1 was unequivocally determined to be based on single crystal X-ray analysis of its precursor 3-acetoxy-6 β -iodo-14-hydroxy-17-cyclopropylmethyl-4,5 α -epoxymorphinan. Both 1 and 2 as well as their corresponding 3-O-acetates were found to readily cross the blood brain barrier and completely reverse the analgesic effects of a 10 mg/kg intraperitoneal dose of morphine sulfate as determined by the paw withdrawal latency test. Compounds 1 and 2 were found to bind with high affinity to μ , δ and κ receptors in vitro. In general, 1 and 2 exhibited higher affinity for μ and κ receptors than naltrexone while the 6 β -iodo epimer 1 (ioxy) was more potent than its epimer 2. In a comparison of the 6 β -halogen substituent on binding affinity across opioid receptor subtypes, it was generally found that I > Br > F. Based on the results of in vitro and in vivo testing, 1 was selected as a target for radiiodination and evaluation as a potential single photon emission computed tomography (SPECT) imaging agent for opioid receptors. Carrier-free [125]1 was synthesized in near quantitative yield by the sequence of reaction of excess 3-acetoxy-6 α -trifluoromethanesulfonyloxy-14-hydroxy-17-cyclopropylmethyl-4,5 α -epoxymorphinan with anhydrous Na 125 I in dry acetonitrile for 90 min at 76 °C followed by deacetylation of the product with 1:1 aqueous ammonia/acetonitrile at 25 °C. The [125]1 has potential as an in vivo imaging agent for opioid receptors.

Ligand Binding Data for Subtypes of the Delta Opioid Receptor- Delta opioid binding sites were assayed using [3 H][D-Ala $_2$,D-Leu $_5$]enkephalin and rat brain membranes depleted of μ binding sites

with the site-directed acylating agent, 2-(*p*-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanatobenzimidazole-HCl. [D-Pen₂,D-Pen₅]enkephalin (DPDPE), [D-Pen₂,L-Pen₅]enkephalin, [D-Ala₂]deltorphin-1 and [D-Ala₂]deltorphin-11 inhibition curves were characterized by slope factors (Hill coefficients) less than 1. The low slope factor of DPDPE persisted in the presence of 50 μ M 5'-guanylylimidodiphosphate in the assay. Quantitative analysis of [D-Ala₂,D-Leu₅]enkephalin, DPDPE and [D-Ala₂]deltorphin-1 binding surfaces resolved two binding sites. Whereas [D-Ala₂,D-Leu₅]enkephalin had equal affinity for both sites, DPDPE and [D-Ala₂]deltorphin-1 had high affinity for the high capacity binding site, and low affinity for the low capacity binding site. These data support pharmacological studies demonstrating δ receptor subtypes which mediate antinociception.

RTI-4614: A Highly Selective Ligand and a Pseudoirreversible Inhibitor of the μ -Opioid Receptor - (\pm)-cis-N-[1-(2-Hydroxy-2-phenylethyl)-3-methyl-4-piperidyl]-N-phenyl propanamide HCl (RTI-4614) is an analog of 3-methylfentanyl, and is a mixture of the 4 possible diastereomers. The apparent K_i values ($nM \pm SD$) of RTI-4614 for μ , δ , κ_1 , κ_2 and κ_3 binding sites were 0.0055 ± 0.0006 , 148 ± 11 , 84.9 ± 12.1 , 2275 ± 147 and 22.3 ± 2.2 nM, respectively, indicating a μ/δ selectivity of about 27,000-fold, and a μ/κ_2 selectivity of about 4000-fold. In other experiments, rat brain membranes were preincubated with concentrations of RTI-4614, washed extensively by centrifugation, and wash-resistant inhibition measured using [³H]DAMGO. Residual drug in the aqueous phase of the membrane suspension was measured by centrifuging the membrane suspension, and assaying the supernatant for inhibitory activity. Receptor inhibition was calculated as wash-resistant inhibition minus supernatant inhibition. The results demonstrated an IC₅₀ for wash-resistant inhibition of 18.7 nM, and a maximal receptor inhibition of about 40%. Saturation binding studies using control and RTI-4614-pretreated membranes demonstrated a decrease in the B_{max} and no change in the K_d. Viewed collectively, these data show that RTI-4614 is a potent and selective agent for the μ receptor, where it can act as a pseudoirreversible binder. Future studies will examine the individual properties of its 4 diastereomers.

Examination of the Possible Mediation of Antineoplastic Effects of Opiates Through Inhibition of Tyrosine-specific Protein Kinases. The possible mediation of opiates as antineoplastics was examined through the inhibition of tyrosine-specific protein kinases. Representative examples of the major classes of opioid nuclei included epoxymorphinans [($-$)-morphine, ($+$)-morphine, codeine and naloxone]; endoetheno-tetrahydrooripavines [buprenorphine and etorphine]; morphinans [levorphanol and dextrophan]; a benzomorphan [(\pm)-phenazocine] as well as the anilidopiperidine, fentanyl and the benzimidazole, etonitazene. With the exception of (\pm)-phenazocine, none of these compounds significantly inhibited the autophosphorylation of EGFR up to concentrations of 1000 μ M. (\pm)-Phenazocine itself exhibited marked inhibition (IC₅₀ = 12 μ M) in the range of the potent tyrosine kinase inhibitor erbstatin which was run as a control in these assays (IC₅₀ = 5 μ M).

Drug Testing Program of the College On Problems Of Drug Dependence. The College (formerly, Committee) on Problems of Drug Dependence (CPDD) and its Drug Evaluation Committee (DEC), have for many years been involved with drug abuse research and the determination of the physical dependence potential and abuse liability of analgesics, stimulants and depressants. For example, potent narcotic antagonists, prepared as precursors of potential imaging agents for SPECT (Single Photon Emission Computed Tomography) scanning were examined for their physical dependence potential and abuse liability, as was LAAM (levo-alpha-acetylmethadol), which is being evaluated as a treatment modality for heroin addicts. The ($+$)- and ($-$)-isomers of N-alkylbenzomorphans were also examined. Some of the ($+$)-enantiomers of these compounds are among the most potent σ ligands known. A considerable number of new fentanyl analogues were investigated and their antinociceptive potency was found to vary from morphine-like to many orders of magnitude more potent than morphine. The potent fentanyl-like compounds appeared to have the physical dependence potential of morphine. Zolpidem (N,N,6-trimethyl-2-(4-methylphenyl)imidazo[1,2-a]pyridine-3-acetamide) tartrate, a hypnotic, was among the stimulant/depressant-type of compounds evaluated and the work of the DEC allowed the prediction that its subjective effects would be similar to those of pentobarbital. This drug is marketed in Europe and was recent reviewed for scheduling in the U.S. Data provided by the DEC are available for scheduling decisions by U.S. federal agencies and the World Health Organization.

Immunoregulatory Opioids

Biochemical and Functional Characterization of a Mu Opioid Receptor Binding Site on Cells of the Immune System - Opioid compounds, such as morphine and β -endorphin, are active immunoregulatory molecules in vitro and in vivo. Many of the effects of these compounds are antagonized by the opioid antagonist naloxone, indicating that opioid receptors are present on immune cells. In this report, lymphocyte μ -opioid receptors were characterized functionally and biochemically using the site-directed acylating agent 2-(p-ethoxybenzyl)-1-[N,N-diethylamino]-ethyl-5-isothiocyanato benzimidazole (BIT) and the μ -selective opioid receptor ligand [D-Ala₂,Me-Phe₄,Gly₅-ol]-enkephalin (DAGO). As determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the lymphocyte μ -class binding site has a molecular weight of 58 kDa under nonreducing conditions and 70 kDa under reducing conditions. By comparison, brain μ -class binding sites labeled with [³H]BIT have a molecular weight of 54 kDa under nonreducing conditions. The BIT-sensitive opioid binding site on lymphocytes is selective for μ -class opioid ligands with noted exceptions. In addition, the site is saturable with between 3,000 and 30,000 sites/cell. Furthermore, the receptor is coupled to calcium uptake pathways in T-lymphocytes, but not in B-lymphocytes. In biological assays, the μ -selective opioid ligands enhance mitogen-induced lymphocyte proliferation, yet suppress natural killer activity. Collectively, the results indicate the presence of a functional μ -type opioid receptor on cells of the immune system and further add to the concept of bidirectional circuitry between the immune and neuroendocrine systems.

Studies Towards the Development of a Cocaine Antagonist

GBR12909 Antagonizes the Ability of Cocaine to Elevate Extracellular Levels of Dopamine - Rats were administered various i.p. doses of the high affinity dopamine (DA) reuptake inhibitor 1-[2-bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine (GBR12909). The caudate nuclei were removed 60 min after drug administration and stored at -70 °C. Striatal membranes were prepared later. The results demonstrated that GBR12909 produced a dose-dependent decrease in the binding of [³H]cocaine or [³H]GBR12935 to the DA transporter (ED₅₀ about 10 mg/kg). Saturation binding studies with [³H]GBR12935 showed that this was due to both an increase in the K_d, due to residual drug, and to a decrease in the B_{max}. At a dose of 25 mg/kg i.p., GBR12909 produced a 50% decrease in the B_{max}, and a 3.4-fold increase in the K_d. In the in vivo microdialysis studies, GBR12909 (25 mg/kg i.p.) produced a modest, long-lasting and stable elevation of extracellular DA. Administration of cocaine through the microdialysis probe to rats pretreated with either saline or GBR12909 (25 mg/kg i.p.) produced a dose-dependent increase in extracellular DA in both groups. GBR12909 inhibited cocaine-induced increases in extracellular DA by about 50% at all doses. These data collectively indicate that at a dose sufficient to decrease by 50% the B_{max} of [³H]GBR12935 binding sites, GBR12909 antagonizes the ability of cocaine to elevate extracellular DA by 50%. Further studies will be needed to evaluate a possible role for GBR12909 in the medical treatment of cocaine addiction.

Evidence for Multiple [³H]GBR12935 Binding Sites Associated with the Dopamine Transporter in Rat Striatal Membranes - A number of radiolabeled ligands (e.g. [³H]GBR12935, [³H]mazindol, [³H]CFT and [³H]BTCF) were used to label the dopamine (DA) transporter, which is implicated in mediating the rewarding effects of drugs. The main objective of the present study was to test the hypothesis that there exist multiple transporter binding sites by conducting quantitative binding studies with each of the [³H]ligands using the same experimental conditions. We report here the results obtained with [³H]GBR12935 and [³H]mazindol. Rat striatal membranes were prepared by two methods: (i) using caudates dissected from completely thawed frozen rat brain and homogenized while thawed (membrane A), and (ii) using caudates homogenized while frozen (membrane B). Two concentrations of each [³H]ligand was each displaced by eight concentrations of nonradioactive drugs: GBR12935, mazindol, CFT and BTCF. This was done for three separate preparations of membrane A and membrane B, generating 216 data points per [³H]ligand and membrane type. Nonlinear least squares curve fitting demonstrated that both the [³H]GBR12935 and [³H]mazindol data sets were best

described by a two site binding model. Although [3 H]mazindol site 2 corresponded to [3 H]GBR12935 site 1, [3 H]mazindol site 1 did not correspond to [3 H]GBR12935 site 2. Qualitatively similar results were obtained with membrane A and membrane B. Control studies demonstrated that [3 H]GBR12935 was not labeling either the piperazine acceptor site or the sigma binding site. Viewed collectively, these studies suggest that in addition to labeling a common binding site/state, [3 H]GBR12935 and [3 H]mazindol each label a distinct binding site/state. Additional independent data will be needed to confirm these findings.

Preliminary Evidence that GBR12909 is Less Effective at Elevating Mesolimbic Dopamine Function than Cocaine - Administration of dopamine (DA) uptake inhibitors to rats stimulates locomotor activity via elevation of mesolimbic DA levels. In the present study, rats received i.p. injections of doses of cocaine (COC, 20 mg/kg), GBR12909 (GBR, 20 mg/kg), nomifensine (NOM, 5 mg/kg), WIN-065-2 (WIN, 1 mg/kg) or saline (SAL) which preliminary studies indicated produce the same level of locomotor stimulation and stereotypy. These parameters were measured for 30 min. At the end of the 30 min test period, the subjects were sacrificed, and the brains removed and kept frozen at -70 °C. The brains were homogenized in 10 ml/gm wet weight ice-cold 10 μ M TRIS-HCl, pH 7.0. The homogenates were then centrifuged at 30,000 x g for 20 min, and the supernatants kept frozen at -70 °C. The next day, aliquots of the supernatants were serially diluted, and assayed for inhibition of [3 H]DA reuptake by a striatal synaptosomal preparation. Only supernatant prepared from GBR-injected rats strongly inhibited [3 H]DA reuptake (IC₅₀ about 100 μ l). The rank-order of potency was GBR>>NOM>WIN=COC. The brain level of GBR was calculated to be about 1 μ M. Control studies determined that brain supernatant did not metabolize these agents. In vivo binding studies using [3 H]BTCP to measure occupancy of the DA transporter resulted in the following rank-order of occupancy: GBR>>NOM>WIN=COC. Since COC produces the same level of response as does GBR, but at lower receptor occupancy, these data support the hypothesis that GBR is less efficacious than is COC at increasing mesolimbic DA function, and are consistent with our previous finding that GBR attenuates the ability of COC to elevate extracellular levels of DA.

Chronic Administration of GBR12909 Partially Attenuates Cocaine-Induced Locomotor Activity in Rats - This study examined the effect of chronic administration of GBR12909 on cocaine-induced locomotor activity. Four groups of male Sprague-Dawley rats were used: VEH/SAL, VEH/COC, GBR/SAL and GBR/COC [COC=cocaine, GBR=GBR12909, VEH=vehicle, SAL=saline]. Rats received daily injections of GBR (10 mg/kg i.p.) or vehicle for 13 days between 9 and 10 AM, followed by administration of either COC (20 mg/kg, i.p.) or SAL 15 min later. Baseline locomotor activity produced by administration of COC or SAL was determined on day #2 (2 days prior to administration of GBR). Locomotor activity was also measured on day #4 and #13 (after 4 and 13 days of administration of GBR or VEH). On day #3, the VEH/COC, GBR/SAL and GBR/COC groups showed essentially the same locomotor activity, which was not significantly different from the baseline measurements. The locomotor activity present in the GBR/COC group was not different from that of the VEH/COC group. On day #13, however, the locomotor activity of the GBR/COC group was significantly reduced by 43% relative to the VEH/COC group. Intense stereotypy was not observed in any of the 4 groups. Interestingly, administration of COC 15 min after GBR did not produce any additional increase in locomotor activity or stereotypy. In a related study, administration of COC (20 mg/kg i.p.) 15 min after administration of COC (20 mg/kg i.p.) also did not produce increased locomotor activity. These data suggest that chronic treatment with GBR may act to attenuate the locomotor effects of COC. Future studies will examine this point using s.q. implantation of GBR pellets.

[3 H]Cocaine Labels a Binding Site Associated with the Serotonin Transporter in Guinea Pig Brain: Allosteric Modulation by Paroxetine - We studied the characteristics of [3 H]cocaine binding to membranes prepared from whole guinea pig brain. Cocaine binding was specific and saturable. A one-site binding model fit the data adequately: the K_d value of [3 H]cocaine was 44 nM with a B_{max} value of 280 fmol/mg protein. The rank order of potency for the [3 H]cocaine binding site was paroxetine > clomipramine > (-)-cocaine > fluoxetine > mazindol > desipramine > GBR12909 > phencyclidine > benztropine > GBR12935 > (+)-cocaine. The IC₅₀ values of these drugs for inhibition of [3 H]cocaine binding were highly correlated with their IC₅₀ values for inhibition of [3 H]5-HT uptake into

synaptosomes prepared from whole guinea pig brain. High affinity 5-HT uptake inhibitors produced dose-dependent wash-resistant (pseudoirreversible) inhibition of [³H]cocaine binding. The wash-resistant inhibition produced by paroxetine was due to an increase in the K_d of [³H]cocaine binding sites, and was accompanied by an increase in the dissociation rate, consistent with an allosteric mechanism. These studies suggest that, using membranes prepared from whole guinea pig brain, [³H]cocaine labels a binding site associated with serotonin transporter and that paroxetine and cocaine bind to different sites on the serotonin transporter.

Synthetic Approaches to Isomeric Isothiocyanato-N-[1-(2-benzo[b]thienyl)cyclohexyl]piperidines. Potential Irreversible Ligands for the Dopamine Transporter - Isomeric isothiocyanate derivatives of the potent dopamine reuptake (DA) inhibitor N-[1-(2-benzo[b]thienyl)cyclohexyl]piperidine (BTCP, 1) were synthesized as potential irreversible ligands for this site. NaNO₂/CF₃CO₂H provided a mild procedure for mononitration of the benzo[b]thienyl ring of 1 as a route to arylisothiocyanates. Novel methodology, utilizing 3,3-ethylenedioxypentane-1,5-diol dimethanesulfonate ester was developed for the synthesis of a precursor for 4-isothiocyanatopiperidine. NaBH₄ or LiAlH₄ reduction of 4-[2-(benzo[b]thienyl)-4-hydroxycyclohexanone and 4-[2-(benzo[b]thienyl)]-4-[1-(piperidinyl)]cyclohexanone oxime gives the corresponding cis diol and cis-1,4-diaminocyclohexane as the major isomers which were investigated as precursors to the cyclohexane ring isothiocyanates. Alternative routes to these isothiocyanates were compared and their stereochemical outcome investigated.

Phencyclidine Recognition Sites

We have studied the action of phencyclidine (PCP, 1-(1-phenylcyclohexyl)piperidine)-like ligands on glutamate receptors of the N-methyl-D-aspartate (NMDA) type. Phencyclidine binding sites have been implicated as allosteric sites which interact with glutamate receptors of the NMDA type. Some phencyclidine (PCP)-like compounds have recently been reported to exert a robust protective effect against neuronal degeneration in ischemia models; evidence suggests they act as antagonists against the depolarizing action of NMDA in animal brain. Other sites for interaction with PCP-like ligands in the CNS have also been found, including the dopamine uptake complex.

The 1-(1-Phenyl-(2-, 3-, and 4-methylcyclohexyl)piperidines Revisited: Synthesis, Stereochemistry, Absolute Configuration, Computer Assisted Molecular Modeling and Biological Effects. The binding affinities, in vivo activities, and absolute configuration (where applicable) of the isomeric 2-, 3-, and 4-methyl substituted PCP isomers were compared with PCP and dizocilpine. The in vitro affinities of the compounds were in reasonable accord with their in vivo activities. In order to see whether the activities of these compounds could be related to their configuration and conformation (their shape in three-dimensional space), we examined and compared them using computer assisted molecular modeling (CAMP). To rationalize the difference in the abilities of the various conformers to interact with the PCP pharmacophore, a simplifying assumption was made. The phenyl ring in PCP-like compounds was assumed to interact with the binding site only when it is in the axial conformation, in agreement with nmr and x-ray crystallography. We have found that the most potent compound in our series, the *trans* 2-methyl compound, exists predominantly with the phenyl moiety in the axial conformation, by nmr (¹³C and ¹H) spectroscopic determinations as well as by x-ray crystallography of the absolute configuration of the molecule. CAMP calculations were in agreement with the experimental data on the *trans* 2-methyl compound. The minimum energy conformer exists predominantly (>99.9%) with the phenyl moiety in the axial conformation. A ca. 6 kcal/mole difference in energy exists between the phenyl axial and equatorial conformers (a difference in 1 kcal/mole corresponds to an 86:14 ratio of conformers). Where our conclusions on the relative potency of a compound from the CAMP calculations were incorrect, we invoked the possibility that the methyl group has intruded into "forbidden" space, that area of space needed by the macromolecule for interaction with the ligand, and thus compounds which were expected to be potent could not attain that potency because of steric overlap. Our results on these PCP-like compounds have led us to conclude that CAMP can be useful for determining the relative activities of new molecules, based on least squares fit to the pharmacophore and calculation of the stability of the minimum energy conformer

with the phenyl axial orientation. The determination of the spatial area required by the macromolecule involved in binding would enable more accurate prediction of the activity of new ligands.

Synthesis and Biological Evaluation of New Conformationally Restricted PCP Analogs. PCP has been found to act as a noncompetitive antagonist for the NMDA receptor. This receptor is involved in normal neuronal functions, as well as in excitotoxicity and neurodegenerative diseases. In our program for the delineation of the structure and function of the PCP binding site on the NMDA receptor, we have recently concentrated on the synthesis and biological evaluation of enantiomeric methyl substituted PCP derivatives. Based on the conformations and binding affinities of these compounds, we have constructed a model of the PCP binding site. More information about the structural requirements of the binding site can be obtained from conformationally restricted analogs of PCP. For example, we see in the literature that methano- and ethano-bridged analogs of PCP show higher affinity for the PCP binding site than PCP, while the adamantyl derivative, which combines both the Phax and Pheq conformations of the phenylcyclohexyl moiety, was found to be devoid of affinity. In contrast, we have replaced the cyclohexane ring with a bicyclo[3.1.0]hexane moiety which restricts the conformational freedom, without introducing additional steric bulk. The affinity of these compounds for the PCP binding site (vs [^3H]TCP) ranges between 0.6 and 29 μM (PCP 0.065 μM). The low affinity can be explained by the preference for the Pheq conformation (cis II), steric interaction of the cyclopropane ring with the binding site (trans I and trans II). Trans-I has been shown to exist in a pseudo-boat conformation (X-ray, courtesy of C. George, NRL, Washington, D.C.), which may not be recognized by the PCP binding site. Furthermore, I and II can be considered as substituted analogs of 1-(1-phenyl cyclopentyl)piperidine, which has significantly lower affinity than PCP for the PCP binding site.

Computer-Assisted Molecular Modeling of the PCP Binding Site Based on Alkyl-Substituted PCP Derivatives. PCP can occur in conformations with the phenyl ring either in axial (Phax) or in equatorial position (Pheq). Based on structure-activity and conformational studies, it can be concluded that Phax is the preferred conformation under most conditions, and probably also the receptor-bound conformation. Introduction of methyl groups in the three rings of PCP affords a series of compounds whose affinity for the PCP binding site (labeled by [^3H]TCP) ranges from 11 to 6800 nM (PCP 65 nM, MK-801 7 nM). Using computer-assisted molecular modeling, we have studied the theoretical conformations of the methyl analogs, and compared them with the preferred conformation of PCP. The methyl analogs can be divided into two groups, based on the stability of the Phax conformation, relative to PCP. Using the "active analog approach", derivatives, for which the Phax was stabilized relative to PCP but which showed lower affinity for the PCP binding site than PCP, were used to describe the receptor-essential volume of the PCP binding site. Similarly, data on a series of monomethyl derivatives of the conformationally rigid MK-801 obtained by Leeson et al., were used. The resulting receptor-essential volumes partly overlap and are complementary to each other. Dexoadrol and etoadrol both show some interaction with the receptor-essential volume, which may explain their lower affinity compared to PCP.

Anticonvulsant Effects of Phencyclidine and PCP-Like Drugs on Audiogenic Seizures Induced by Metaphit in Mice. (+)- and (-)-MK-801 improved delayed amnesia, but the effects of phencyclidine were weak. These NMDA antagonists protected CA1 hippocampal neurons from the morphological changes (swelling) induced by CO. Our preliminary results show that the effects of NMDA antagonists are not related to their actions on body temperature. On the contrary, (+)-MK-801 and phencyclidine potentiated acute amnesia, but (-)-MK-801 did not. It is suggested that there is a stereoselectivity in the effects of MK-801 on CO induced amnesia, and that CO induced delayed amnesia can be used as an ischemic animal model.

Effect of Metaphit on Dopaminergic Neurotransmission in Rat Striatal Slices: Involvement of the Dopamine Transporter and Voltage-Dependent Sodium Channel. Metaphit, an isothiocyanate analog of phencyclidine (PCP), increased the basal release of radioactivity (outflow) from perfused rat striatal slices preloaded with [^3H]dopamine above levels observed with the dopamine uptake blocker nomifensin. Preperfusing the slices with metaphit, followed by its removal, attenuated the amphetamine- or dopamine-induced outflow. In slices prepared from reserpine-pretreated rats, the metaphit (100 μM)-induced outflow was reduced to that observed with 10 μM nomifensin, suggesting a

vesicular releasing effect of metaphit in addition to dopamine uptake blockade. Electrically induced overflow of radioactivity from normal slices was stimulated by nomifensin and PCP, and by metaphit at 3 μ M; it was unaffected by metaphit at 10 and 25 μ M, and inhibited by higher concentrations of metaphit. Evidence that the latter effect is due to blockade of voltage-dependent sodium channels is as follows. First, metaphit, as did PCP, inhibited the binding of [3 H]batrachotoxinin A 20-a benzoate to rat striatal synaptoneurosome by increasing its dissociation rate; the effect of PCP, but not that of metaphit, was reversible by washing. Second, metaphit, as did PCP, inhibited veratridine (5 μ M)-induced influx of [14 C]guanidinium ion into synaptoneurosome. Third, metaphit inhibited overflow of radioactivity from [3 H]dopamine-preloaded slices induced by 2.5 μ M veratridine, as did the sodium channel blocker tetrodotoxin.

Electroencephalographic Characteristics of Audiogenic Seizures Induced in Metaphit-Treated Small Rodents. Adult male mice, rats, and guinea pigs were subjected to intense sound stimulation of an electric bell (100 dB, 12 kHz for 60 s) after a single intraperitoneal (i.p.) injection of metaphit (1-(1-(3-isothiocyanatophenyl)cyclohexyl)piperidine) (50 mg/kg). When the animals were tested 24 h after administration of metaphit, audiogenic seizures were observed. None of the control saline-injected animals had convulsions. EEG recordings demonstrated the appearance of paroxysmal activity and spike-wave complexes in the trace from cortical and hippocampal electrodes, with frequency and amplitude increasing with time. Behaviorally, myoclonic jerks of facial muscles, ears, and neck appeared, but no correlation was noted between EEG and the motor phenomena. Auditory stimulation was necessary to elicit the full-blown sequence of seizure responses consisting of wild running followed by clonic and then tonic extension. At the time of seizures, repetitive high-amplitude spikes and waves appeared in the EEG, followed by profound EEG and behavioral depression. None of the animals died during or immediately after seizures. The seizure response to sound stimulation of mice, rats, and guinea pigs was phenomenologically similar, with minor differences in quantitative pattern of convulsive components, which suggests that all three animal species share the common property of extreme susceptibility to audiogenic stimulation caused by metaphit administration.

Analogues of the Dioxolanes Dexoxadrol and Ettoxadrol as Potential Phencyclidine-like Agents. Synthesis and Structure-Activity Relationships. A series of dioxolane analogues based on dexoxadrol (4S,6S-2,2-diphenyl-4-(2-piperidyl)-1,3-dioxolane) and ettoxadrol (2S,4S,6S-2-ethyl-2-phenyl-4-(2-piperidyl)-1,3-dioxolane) were prepared and tested for their ability to displace [3 H]TCP (1-[1-(2-thienyl)cyclohexyl]piperidine) from PCP (1-phenylcyclohexyl)piperidine binding sites in rat brain tissue homogenates. Qualitative structure-activity relationships within this series were explored through modifications of the three major structural units of dexoxadrol, the piperidine, 1,3-dioxolane and aromatic rings of the molecule. N-Alkyl derivatives of dexoxadrol were found to be inactive, as were those analogues where the dioxolane ring was modified. Phenyl-substituted ettoxadrol analogues were compared to similarly substituted PCP analogues and distinct differences were found in their structure-activity relationships suggesting that the aromatic rings in these two drug classes interact differently with the PCP binding sites. The replacement of the phenyl ring in ettoxadrol by either a 2- or 3-thienyl ring led to compounds with affinity comparable to ettoxadrol, and the replacement of the ethyl moiety on ettoxadrol's dioxolane ring with propyl or isopropyl led to compounds which were more potent than ettoxadrol or PCP. The most potent compound was 2S,4S,6S-2-ethyl-2(1-chlorophenyl)-4-(2-piperidyl)-1,3-dioxolane, where a chlorine moiety was placed in the ortho position in the aromatic ring of ettoxadrol. Its potency was comparable with TCP in vitro.

Anticonvulsant Activity of the Low Affinity Uncompetitive NMDA Agonist Racemic 5-Aminocarbonyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (ADCI): Comparison with the Structural Analogs Dizocilpine (MK-801) and Carbamazepine. (\pm)-5-Aminocarbonyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (ADCI), a tricyclic compound structurally related to dizocilpine (MK-801) and carbamazepine, was a potent anticonvulsant in the mouse maximal electroshock seizure test when administered i.p. (ED₅₀, 8.9 mg/kg) or p.o. (ED₅₀, 23.5 mg/kg), but failed to cause motor impairment except at substantially higher doses (TD₅₀ values, 49.2 mg/kg i.p. and 293 mg/kg p.o.). ADCI was also protective against chemically induced seizures in mice, including those produced by 4-aminopyridine (ED₅₀, 7.1 mg/kg s.c.) and pentylenetetrazol (ED₅₀, 37.4 mg/kg s.c.). In addition, ADCI antagonized the behavioral effects and lethality of s.c. administered N-methyl-D-aspartate (NMDA; ED₅₀, 15.2 mg/kg), but was a weaker antagonist of kainate-induced clonic

seizures (ED₅₀, 33.0 mg/kg), indicating that the drug is a selective functional NMDA antagonist. In common with other NMDA antagonists, ADCI retarded the development of amygdaloid kindled seizures in rats, but failed to attenuate the afterdischarge duration in fully kindled animals. Whole cell voltage clamp recordings from cultured hippocampal neurons demonstrated that ADCI selectively blocks inward current responses to NMDA in a use dependent fashion without affecting responses to kainate or quisqualate, indicating that ADCI is a selective open channel (uncompetitive) blocker of the NMDA receptor-ionophore complex. ADCI blocked NMDA-evoked inward current responses with a potency (IC₅₀, 14 mM) similar to that with which it displaces [³H]-1-[1-(2-thienyl)cyclohexyl]piperidine from binding to NMDA receptor channels in rat brain homogenates (IC₅₀, 11.3 μM). In contrast, dizocilpine (MK-801) was a high potency antagonist of NMDA responses (IC₅₀, 10 nM) whereas carbamazepine only minimally affected NMDA responses even at high concentrations (IC₅₀ > 300 mM). We conclude that ADCI is a low-affinity uncompetitive NMDA antagonist which, like other NMDA antagonists, has a broad spectrum of anticonvulsant activity in animal seizure models. However, in contrast to conventional (high affinity) NMDA antagonists whose propensity to cause neurological side effects may limit their therapeutic usefulness, ADCI has a therapeutic index [maximal electroshock ED₅₀/TD₅₀ 5.5 (i.p.) or 12.5 (p.o.) in the mouse] comparable to that of the widely used antiepileptic drug carbamazepine.

MPTP Lesions of the Nigrostriatal Dopaminergic Projection Decrease [³H]1-[1-(2-Thienyl)Cyclohexyl]Piperidine Binding to PCP Site 2: Further Evidence that PCP Site 2 is Associated with the Biogenic Amine Reuptake Complex. Our previous studies have demonstrated that, using membranes of guinea pig brain, [³H]TCP labels not only the phencyclidine binding site associated with the NMDA receptor (PCP site 1), but also a second high affinity binding site which is associated with the biogenic amine reuptake carrier (termed PCP site 2). To test this hypothesis, the binding of [³H]GBR12935 to the dopamine transporter, and [³H]TCP binding to PCP sites 1 and 2 were measured in caudates harvested from control, MPTP-treated and reserpine-treated dogs. MPTP treatment decreased dopamine levels by over 99%, decreased [³H]GBR12935 binding by over 90%, decreased [³H]TCP binding to PCP site 2 by about 50%, and had no significant effect on [³H]TCP binding to PCP site 1. These data are consistent with the hypothesis that a portion of PCP site 2 is associated with dopaminergic nerve terminals in dog caudate.

Structure-Activity Studies on the Interaction of Biogenic Amine Reuptake Inhibitors and Potassium Channel Blockers with MK-801-Sensitive (PCP Site 1) and -Insensitive (PCP Site 2) [³H]TCP Binding Sites in Guinea Pig Brain. Binding of [³H]TCP to guinea pig brain membranes was well described by a two site binding model with K_d values of 13.4 nM and 56.8 nM; the density of the two binding sites were roughly the same. We found that [³H]TCP binding to the higher affinity site (PCP site 1) was displaced by (+)-MK801, a high affinity ligand for the dissociative anesthetic binding site on the NMDA receptor-channel complex, with a K_i value of 1.91 nM, whereas [³H]TCP binding to site 2 was relatively insensitive to (+)-MK801 (2543-fold less selective). In contrast, binding to site 2 was relatively more sensitive to displacement by CMI, an inhibitor of biogenic amine reuptake (13.5-fold selective).

The Competitive NMDA Receptor Antagonist, CPP, Allosterically Modulates the NMDA Receptor Associated Phencyclidine Binding Site in the Apparent Absence of Steric Hindrance. The primary purpose of this study was to determine the mechanism by which CPP inhibits [³H]TCP and [³H](+)-MK801 binding in crude membranes possessing different levels of non-sequestered GLU and GLY. We conducted several different types of experiments to distinguish among three models: the steric hindrance model, an allosteric model and a competitive model. Unlike the steric hindrance model, the allosteric model does not postulate that the CPP-induced inhibition of [³H]TCP and [³H](+)-MK801 binding results from closure of channels. Rather, the allosteric model suggests that the binding of CPP to the NMDA site produces a conformational change in the PCP site which results in an increase in the K_d of [³H](+)-MK801 or [³H]TCP for the PCP binding site. The competitive model, on the other hand, suggests that CPP binds directly in the PCP site, producing an inhibition of binding. The major findings of this study are: 1) CPP inhibits [³H](+)-MK801 and [³H]TCP binding to the NMDA receptor associated PCP binding site in a dose-dependent manner. 2) This effect is reversed by the addition of

GLU. 3) A steric hindrance model can not explain these data because a) the effect is observed at true equilibrium; b) in the presence of saturating concentrations of CPP (closed channels) the initial rates of [^3H]ligand association are directly proportional to the concentration of [^3H]ligand; and c) the inhibitory effect of CPP is observed in the presence of low levels of non-sequestered endogenous GLU. 4) The data are most simply explained on the basis of an allosteric model which postulates that occupation of the NMDA receptor by CPP alters the conformation of the PCP binding site. 5) The mechanism by which depleting membranes of endogenous GLU results in the observation of steric hindrance might involve factors other than the removal of GLU.

Cannabinoid Receptors

Neuronal Localization of Cannabinoid Receptors in the Basal Ganglia of the Rat - Cannabinoid receptors have recently been characterized and localized using a high-affinity radiolabeled cannabinoid analog in section binding assays. In rat brain, the highest receptor densities are in the globus pallidus and substantia nigra pars reticulata. Receptors are also dense in the caudate-putamen. In order to determine the neuronal localization of these receptors, selective lesions of key striatal afferent and efferent systems were made. Striatal neurons and efferent projections were selectively destroyed by unilateral infusion of ibotenic acid into the caudate-putamen. The nigrostriatal pathway was selectively destroyed in another set of animals by infusion of 6-hydroxydopamine into the medial forebrain bundle. After 2- or 4 week survivals, slide-mounted brain sections were incubated with ligands selective for cannabinoid ([^3H]CP 55,940), dopamine D_1 ([^3H]SCH-23390) and D_2 ([^3H]raclopride) receptors, and dopamine uptake sites ([^3H]GBR-12935). Slides were exposed to ^3H -sensitive film. The resulting autoradiography showed ibotenate-induced losses of cannabinoid, D_1 and D_2 receptors in the caudate-putamen and topographic losses of cannabinoid and D_1 receptors in the globus pallidus, entopeduncular nucleus, and substantia nigra pars reticulata at both survivals. Four weeks after medial forebrain bundle lesions (which resulted in amphetamine-induced rotations), there was loss of dopamine uptake sites in the striatum and substantia nigra pars compacta but no change in cannabinoid receptor binding. The data show that cannabinoid receptors in the basal ganglia are neuronally located on striatal projection neurons, including their neurons and terminals. Cannabinoid receptors may be co-localized with D_1 receptors on striatonigral neurons. Cannabinoid receptors are not localized on dopaminergic nigrostriatal cell bodies or terminals.

Sigma Receptor Ligands

Sigma receptors are non-dopaminergic, non-opioid receptors which bind PCP- and D_2 -dopamine antagonist-related compounds, as well as some of the (+)-enantiomers of the morphinan and benzomorphan opioids. Studies utilizing various sigma ligands have implicated sigma receptors in neural regulation of motor behavior and modulation of transmitter release upon electrical stimulation of smooth muscle preparations. Although progress is being made in elucidating the physiological roles of sigma receptors, the biochemical systems modulated by sigma receptors are not clear.

Characterization of the Enantiomers of *cis*-N-[2-(3,4-Dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)cyclohexylamine. Novel Compounds with High Affinity, Selectivity and Biological Efficacy at Sigma Receptors - A novel class of compounds with very high affinity and selectivity for sigma receptors has been discovered. (\pm)-*cis*-N-[2-(3,4-Dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)cyclohexylamine (BD614) and its optically pure 1*S*,2*R*-(-)- (BD737) and 1*R*,2*S*-(+)- (BD738) enantiomers bound to sigma receptors of guinea pig brain with $K_i = 2.0 \pm 0.4$ nM, 1.3 ± 0.3 nM, and 6 ± 3 nM, respectively. These compounds exhibited little or no affinity for dopamine- D_2 , kappa opiate, or phencyclidine receptors and displayed biological efficacy in assays of sigma receptor function: to produce alterations in motor behavior and inhibition of the muscarinic cholinergic phosphoinositide response. Microinjection of BD614 into the rat red nucleus or substantia nigra produced a dose-dependent alteration in head position and contralateral circling, respectively. BD614, BD737, and BD738 inhibited stimulation of inositol phosphate production by carbachol or oxotremorine-M in a dose-dependent manner. Thus, N-substituted *cis*-2-(1-pyrrolidinyl)cyclohexylamines may prove useful in studies of sigma receptor structure and function.

Evaluation of Novel U50,488H Analogs for Antiischemic Activity in the Gerbil - U-50,488H, a kappa (κ) opioid ligand with moderate potency at sigma (σ) receptors, protects against mechanical and ischemia-induced injury. The purpose of this study was to evaluate the possibility that σ -receptors may be involved in mediating the neuroprotective actions of U-50,488H. This possibility was examined by testing the potential of a series of U-50,488H analogs, which are potent σ -ligands with minimal activity at κ -opioid receptors, to protect against ischemia-induced neuronal damage in the gerbil. Like U-50,488H, BD-449 (20 mg/kg), the cis-diastereomer of U-50,488H, protected against ischemia-induced neuronal damage as did BD-737 (50 and 30 mg/kg) and BD-738 (50 mg/kg). All 3 compounds interacted selectively with σ -receptors. In contrast, BD-601 (50 mg/kg), did not protect against ischemia-induced neuronal damage, although it also interacted potently with σ -receptors. One difference between the compounds that were neuroprotective and BD-601 is that only BD-601 produced σ -like behavioral effects in the rat. Thus, it is possible that BD-601 may interact differently or at a different σ -subtype than BD-449, BD-737 and BD-738 with σ -receptors. However, these results clearly indicate that an interaction with κ -opioid receptors is not required for anti-ischemic activity, and that σ -receptors may play a role in neuroprotection.

Behavioral Pharmacology Unit of the Drug Design and Synthesis Section

Behavioral Effects of the Putative Ethanol Antagonist, Or 15-4513 - The effects of the imidazobenzodiazepine, Or 15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo (1,5-a)(1,4) benzodiazepine-3-carboxylate) and ethanol were studied on fixed-ratio 30 (FR 30) responding of mice. Or 15-4513 (0.3-30 mg/kg) decreased FR responding, but the duration of the rate-decreasing effect was relatively short. Following the complete cessation of responding by high doses (30 mg/kg), recovery was rapid and complete within 15-20 min. Ethanol (1-4 g/kg i.p.) and pentobarbital (3-56 mg/kg) also only decreased responding, with the effect lasting considerably longer than that of Or 15-4513. Or 15-4513 (3 mg/kg) attenuated the rate-decreasing effect of ethanol, when given either 1 min before, or 5 min after, single doses of ethanol. A lower dose of Or 15-4513 attenuated the rate-decreasing effects of intermediate doses of ethanol but not pentobarbital. The results confirm that Or 15-4513 can antagonize the rate-decreasing effect of ethanol over a limited range of doses. The results suggest the antagonism depends neither upon the behavioral effect of ethanol nor upon opposing behavioral effects of Or 15-4513.

Behavioral Effects of Stress-related Peptides and Hormones - Previous studies have linked susceptibility to drug abuse with individual differences in susceptibility to stress activation of the hypothalamic pituitary adrenal (HPA) axis. The primary endogenous agents involved in the HPA axis are corticotropin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH) and cortisol (B). Several studies have been directed at examining the behavioral specificity of the direct effects of CRH on behavior. In order to accomplish this, a unique i.c.v. delivery device had to be designed, constructed and tested. This large animal intracerebral drug administration unit is designed to allow the delivery of drugs or other agents to discrete loci within the brains of animals while maintaining sterile conditions. It is an improvement over existing designs because it 1) maintains an absolute minimal dead space within the system, 2) is smaller in diameter (by approximately 80%) than existing shunt catheters, minimizing tissue damage during placement, 3) is easily secured and requires minimal clearance over the cranium, and 4) maintains a sterile seal between the brain and periphery. Preliminary studies indicate the device is well accepted and fully functional for periods up to a year. The device is intended for permanent implantation.

Several additional studies were directed at testing the device. In one, the behavioral consequences of the central administration of corticotropin releasing hormone (CRH) in rhesus monkeys was determined using food-maintained behavior. Acute doses of CRH (0.003 ng/kg-10 μ g/kg, i.c.v.), decreased responding for food in a dose- and time-related manner. With intermediate doses, responding occurred at a high rate until food was delivered, and then abruptly ceased for several minutes. Previous studies have attributed similar effects to the noxious properties of certain drugs. Acute doses had no effect on home cage food consumption, body weight, or responding for food on subsequent days. When CRH was given repeatedly for several days, its behavioral suppressant effects increased. Home cage food intake, body weight, and subsequent responding for food decreased for up to six weeks before returning to normal. These results suggest that sustained elevations in central levels of CRH can result in a sensitization to its

anorexiogenic effects, an effect that has not been reported in other species. Because hyper-aroused clinical states such as depression, anorexia nervosa, and some forms of drug abuse are characterized biochemically by hypercortisolism and elevated CRH in CSF, these anorexiogenic effects may corroborate a potential role for CRH in affective disorders where comorbidity with drug abuse is high.

In another study rhesus monkeys were equipped with the i.c.v. cannula system and trained to respond under operant schedules of food presentation or from stimuli associated with the delivery of shock (escape). CRH decreased food-maintained behavior in a dose-related manner over the range of (0.3-10 $\mu\text{g/kg}$) but did not affect escape responding, demonstrating a selective effect on food-maintained responding. This selective effect was related to the tendency for responding to stop after delivery of a food pellet when higher doses of CRH were given, consistent with the notion that a conditioned aversion to food was established in the presence of CRH. This may suggest that in hyper-aroused clinical states such as depression and anorexia nervosa, focus is shifted away from appetitive tasks as a result of increased levels of CRH.

Several studies directed at determining the nature of this behavioral specificity of the effects of the CRH peptide were also conducted, and incorporated into a recently published review. This review focuses on those aspects of the behavioral effects of CRH related to food-associated behaviors. The effects of CRH on food intake are compared with its effects on performances maintained by food presentation, and contrasted with the effects of CRH on performances maintained by other events. The effects of CRH antagonists and drugs which interact with the behavioral effects of CRH are also reviewed, particularly with respect to their direct effects on food intake. Lastly, data assessing the effects of CRH administration on central neurotransmitter levels are presented and compared with levels seen in clinical populations. The effect of CRH on food intake seen in animals is consistent with a putative role for CRH in clinical syndromes where appetite suppression is apparent. Since some of the effects of CRH on food intake are subject to pharmacological intervention, strategies directed at peptidergic mechanisms of drug-abuse associated disorders should be explored.

Several additional behavioral studies were directed at the association of the particular effect a drug of abuse, such as cocaine, can have on subsequent tendencies to abuse or avoid further drug contact. In one set of experiments squirrel monkeys were trained to respond under second-order schedules of food presentation and then exposed to either a self-administration (SA) or to a conditioned taste aversion (CTA) procedure. Initial exposure to stimuli associated with post-session administration of 0.3 mg/kg cocaine either maintained (SA) or suppressed (CTA) responding, respectively. The monkeys were then exposed to the alternate procedure. Initial exposure to CTA, decreased cocaine SA responding compared to rates seen with initial SA exposure. In contrast, with initial exposure to SA, the CTA procedure failed to suppress responding. Thus, prior exposure to either reinforcing or suppressant effects of cocaine altered the subsequent behavioral effects of that drug, suggesting a unique role of behavioral history in the abuse potential of cocaine.

Because of the unique behavioral effects of drug delivery scheduled to occur in conjunction with novel food delivery appear to determine specific properties of drugs, some further studies were initiated to investigate these effects in rhesus monkeys. The development of drug discrimination was assessed in rhesus monkeys using the conditioned-taste-aversion paradigm. Monkeys were initially trained to respond under a fixed-ratio 30-response schedule of food-pellet delivery to assess the rate-decreasing effects of alprazolam (0.03-3 mg/kg, i.m., 60 min pre-session). Alprazolam decreased responding at doses greater than 0.1 mg/kg. Discriminative stimulus effects of alprazolam were then assessed by giving 0.03 mg/kg before sessions in which 1.8 mEq/kg lithium chloride was given immediately after the session (alprazolam/lithium session). On intervening days, saline was given before and after the session (saline/saline session). Rates of responding decreased over successive alprazolam/lithium sessions and also during the saline/saline session that immediately followed an alprazolam/lithium session. During subsequent saline/saline sessions, rates of responding returned to levels near baseline rates within 2-4 sessions. The discriminative stimulus effects of alprazolam were then assessed by giving 0.1 mg/kg before sessions in which 1 mg/kg *d*-amphetamine was given immediately after the session (alprazolam/*d*-amphetamine session). Rates of responding decreased during subsequent alprazolam/*d*-amphetamine sessions in drug-experienced monkeys, but not during intervening saline/saline sessions. These findings demonstrate that drug stimuli associated with post-session drug

injections can rapidly develop control over behavior and suggest that similar methods be explored in the assessment of drug-discrimination.

Individual Differences in Pharmacological and/or Behavioral Effects of Treatment in Rat Strains -

Recent studies at the University of Bordeaux and the Addiction Research Center of NIDA have shown that the LEW/N rat strain is considerably more likely to initiate drug taking behavior and to demonstrate behavioral effects of drugs of abuse than are its histocompatible counterpart, the F344/N rat. Several studies have been directed at elucidating the nature of these behavioral differences, in order to associate the range of behavioral markers with specific gene loci being identified in other Institutes. For example, both NIMH and NIAMS have intensive molecular programs to identify genetic differences, with which the PI collaborates. In the initial series of these studies, stress-related differences in these strains was shown with a number of different behavioral stressors, and preliminary evidence that these differences may stem from differences in endogenous corticotropin releasing hormone was provided.

These studies also showed that challenge with inflammatory stimuli, stressors, or specific drugs render LEW/N rats susceptible to autoimmune disease while their histocompatible control, the F344 rat, are resistant. In order to examine behavioral correlates of suspected differences in hypothalamic pituitary adrenal mechanisms responsible for that effect in these strain, both strains were a) assessed for differences in behavioral and corticosterone responses to exposure to an open field, b) prepared with ventricular cannuli, and assessed again in the open field after saline, or c) after 3 µg/rat of CRH. Significant baseline differences in open field response (pattern of ambulation), and in the effects of CRH on rearing, grooming, and activity were found between these strains. These differences suggest that differences in endogenous CRH may form the basis for the differential susceptibility of these strains to autoimmune disease. Such differences may serve as an animal model for genetic determinants of relationships between CNS function and the immune system.

Additional studies designed to further characterize potential behavioral correlates of these differences, the amplitude of the acoustic (ASR) and tactile (TSR) startle response and the corticosterone response to acoustic startle stimuli were compared between two histocompatible strains, Lewis (LEW/N) and Fischer (F344/N) rats, as well as outbred Harlan Sprague-Dawley (SD) rats. Startle stimuli elicited larger ASR and TSR in LEW/N rats than in F344/N rats, with SD rats exhibiting an intermediate response. The ASR habituated at a similar rate in LEW/N and F344 rats, while the ASR did not habituate in SD rats. After handling and placement in the startle chambers, the three strains did not differ in control levels of corticosterone. In contrast, exposure to acoustic startle stimuli increased corticosterone 5-fold in F344/N rats and 2-fold in SD rats, but had no effect on corticosterone in LEW/N rats. These findings suggested an inverse relationship between the amplitude of the ASR and hypothalamic-pituitary-adrenal activation across strains. This relationship was further supported by a high negative correlation between corticosterone level and ASR amplitude within the F344/N group.

Behavioral Toxicology and Risk Assessment - The PI carries a concern for how a variety of different agents found in the workplace, home or as other forms of environmental exposure, can affect normal behavioral functioning. In particular, expertise in the assessment of volatile organic solvents was established at Harvard Medical School before coming to the NIH, but as opportunities arise, the interest is maintained. One line of work has to do with changing current notions of how to predict risks associated with exposures to toxic agents. The PI has maintained a collaboration with the USEPA to design dose-effect based risk assessment methods. Risk assessment is the attempt to characterize the chance of obtaining an adverse effect after exposure to an agent. Traditionally, high levels of an agent have been used to estimate the likelihood a lower dose might have an effect either by using low-dose extrapolation models or by attempting to establish a dose with no observable effects (NOEL). Low-dose extrapolation models yield estimates for small effects, but these estimates may vary by orders of magnitude depending upon the function chosen to represent the data. NOEL's are imprecise because a true no-effect level is indeterminant and the inability to determine an observable effect depends primarily on background variability. Newer methods use data from portions of the dose-effect function where error is smaller to estimate risks. Risk estimates using two of these approaches are compared for two different sample sizes. Each method produced the same estimate with the larger sample at low risk, but with increasing levels of risk and smaller samples the estimates obtained using these methods diverged.

Several studies were completed to develop these methods. In one, four homologous n-alkanes were compared for their ability to impair performance and stimulate hypothalamic-pituitary activity in mice. Performance was assessed using operant responding maintained under an FI 60-sec schedule of milk presentation. Cumulative concentration-effect functions for octane, heptane, hexane and pentane were obtained by incrementally increasing exposure concentrations until responding was abolished. Recovery from these rate-decreasing effects was determined 30 min after exposure to the highest concentration. Rate-decreasing potency (EC50) was greatest for octane (2474 ppm), and progressively less for heptane (3872 ppm), hexane (7051 ppm), and pentane (36130 ppm). Responding recovered completely, 30 min after exposure, for pentane and hexane, to 75% of pre-exposure levels for heptane, but to only 15% of pre-exposure levels for octane. The risk of obtaining a small effect with these agents (the concentration expected to decrease performance 10% in one out of one thousand mice) exhibited a similar order. The effect was predicted to occur at 227 ppm for octane, 331 ppm for heptane, and 1429 ppm for pentane. However, this prediction occurred at an unusually low dose for hexane (68 ppm). These n-alkanes also stimulated up to 2000-fold increases in adrenocorticotropin hormone (ACTH) release. n-Hexane was slightly more potent and produced larger effects. These studies demonstrate a direct relationship between aliphatic carbon chain length and the potency of n-alkanes in impairing performance.

Another study was directed at assessing similar effects in an anesthetic agent, diethyl ether. Diethyl ether has anesthetic, neuroendocrine-stimulating, and abuse-potential properties, yet little is known of the concentrations over which these apparently diverse behavioral effects occur. Adult male NIH mice were exposed to a range of concentrations of ether (1000-30000 ppm) in order to characterize its effects on operant behavior and neuroendocrine activity. When responding was maintained under FI-60 sec schedules of milk presentation, 5- or 30-min exposures to less than 3000 ppm ether were without behavioral effect, 10000 ppm produced up to 300% increases in responding, and higher concentrations abolished responding. Exposure to a similar range of concentrations elevated adrenocorticotrophic hormone (ACTH) and corticosterone levels in a dose- and time-dependent manner. Short (5 min) exposures elevated baseline levels of ACTH (18.2 pg/ml) to 310.5 pg/ml (~1700% of control) at 10000 ppm, whereas corticosterone was relatively unaffected. With 30 min of exposure to 10000 ppm ether, corticosterone increased maximally from 78.44 ng/ml to 559 ng/ml (~700% of control) and ACTH was increased to a lesser extent. The imidazobenzodiazepine, Or 15-4513 decreased FI responding at doses greater than 3 mg/kg and attenuated the rate-increasing effects of diethyl ether at 1 mg/kg.

Behavioral Effects of β -Carbolines - β -Carbolines comprise a class of agents with profound behavioral effects, and may possibly serve as a pharmacological model of stress or anxiety. In order to better characterize its behavioral effects, lever-pressing of squirrel monkeys was maintained under a multiple fixed-interval (FI) 5-min schedule of food presentation. In one component, responding was suppressed to various degrees by the presentation of electric shock following each 30th response. When responding was either substantially or minimally suppressed, intermediate doses of chlordiazepoxide (CDAP, 1-30 mg/kg) increased both suppressed and non-suppressed responding. β -carboline 3-carboxylic acid ethyl ester (β -CCE, 0.1-3 mg/kg) had little effect at low to intermediate doses (0.1-0.3 mg/kg) and decreased both minimally-suppressed and non-suppressed responding to a comparable extent at higher doses. Repeated daily dosing with β -CCE (up to 10 mg/kg) resulted in rapid tolerance to its rate-decreasing effects. As agonists do not typically exhibit rapid tolerance for anxiolytic efficacy, these results suggest that some behavioral effects of inverse agonists may not be strictly opposite those of benzodiazepines.

Effects of Sigma Ligands - Excitatory amino acids, acting at the NMDA receptor, have been postulated to play an important role in the acquisition of behavior (learning). Drugs that block this receptor attenuate many of the effects associated with learning and memory. To determine whether excitatory amino acid blockade prevents the acquisition of a conditioned emotional response (CER), we studied the effects of MK-801, a non-competitive NMDA receptor antagonist, on the development of conditioned response suppression in the rat. In contrast to the rapid development of response suppression over the first eight days of exposure to the procedure in rats treated with saline, responding was not suppressed in rats treated with MK-801. Thus, these data are consistent with the notion that excitatory amino acid blockade prevented the development of a learned emotional response, suggesting a potential role for this receptor in the development of anxiety-related disorders in humans.

Behavioral Consequences of HIV Infection: Role of GP120 in Dementia - The effects of recombinant and purified native gp 120, the envelope protein of human immunodeficiency virus (HIV), were assessed on the acquisition of a spatial discrimination in a Morris water maze. Nanogram levels of gp120 dose-dependently retarded acquisition, suggesting it may play a role in impairing cognitive processes in AIDS-infected individuals. The potential precursor, gp160, had no effect on spatial performance. Since there are several functional homologies between gp120 and vasoactive intestinal polypeptide (VIP), a novel VIP antagonist was also assessed. The VIP antagonists also impaired the acquisition of performance in the maze, and the effect was reversible with VIP at doses that alone had no effect on performance. Thus, gp120 may induce cognitive impairment in HIV-infected individuals, possibly through the interference of VIP-mediated activity in the central nervous system.

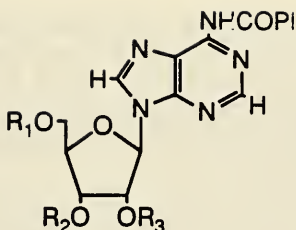
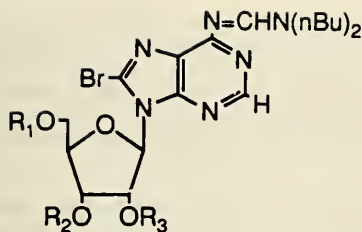
BIOMEDICAL CHEMISTRY SECTION

The Solid Phase Synthesis of 2',5'-Linked Oligoribonucleotides Containing 8-Bromoadenosine - 8-Bromoadenosine and its nucleotides occupy an important place in nucleoside/nucleotide chemistry as substrates for synthesis of many other 8-substituted derivatives either through nucleophilic substitution or UV light induced photolysis. Although they are not highly cytotoxic their biological activity is often different than that of adenosine, and this difference is most probably caused by well established *syn* conformation around glycosidic bond. For example, 8-bromoadenosine is 10^5 times less efficient as substrate for adenosine deaminase, is inactive at adenosine receptors but is phosphorylated by adenosine kinase.

We have studied extensively 2',5'-linked 8-bromoadenylyl oligonucleotides as analogs of 2-5A. 2-5A, consisting of a series of 2',5'-oligoadenylylates of a general formula $ppp5'A_2'(p5'A)_n$, is synthesized in interferon treated cells by the 2',5'-oligoadenylyl synthetase in response to dsRNA and uniquely activates a latent ribonuclease, RNase L, which in turn degrades cellular and/or viral RNA. When compared to 2-5A, brominated analogs were more stable to degradation by 2',5'-specific phosphodiesterase, and analogs containing one or two adenine residues at the 5'-end were bound to RNase L with the same efficiency as the 2-5A and activated the enzyme already as 5'-monophosphates. The efficient binding and photoreactivity of 8-bromoadenine residue allowed their application as a highly sensitive photo-crosslinked probe for detecting RNase L.

The method of synthesis of these sequence specific oligobromoadenylylates which we were initially using was based on controlled stepwise lead-ion catalyzed addition of a corresponding nucleoside 5'-phosphoroimidazolidate to the 2'-OH end of 5'-end protected (as phosphoromorpholidate) nucleoside or oligonucleotide. Because a number of by-products formed after each step product purification had to be performed the procedure was a very time consuming one. During that study it became clear that we needed a better, more efficient way of oligo(bromo)adenylyl synthesis and we decided to determine if the solid-phase method of oligoribonucleotide synthesis could be adapted to include 8-bromoadenylylates. In initial consideration of the proper N^6 protection we eliminated any acyl protecting group. Although N^6 -benzoyl protection of adenylyl residues was successfully used in the solid phase synthesis of oligoribonucleotides, both 2',5'- and 3',5'-linked, 8-bromosubstitution of the purine increases the lability of the purine ring towards alkaline hydrolysis and has a destabilizing effect on the glycosidic bond under acidic conditions. Both of these effects are further enhanced by acylation of exocyclic amino group (Amarnath, V. and VanBoom, A.D. (1977) Chem. Rev. 77, 183). Recent reports on applications of dialkylformamidines as a depurination resistant amino group protection in the solid phase synthesis of oligodeoxynucleotides prompted us to evaluate this group for 8-bromoadenosine.

Several differently substituted amidine derivatives of deoxynucleosides (dAdo, dCyd, dGuo) have been synthesized and applied to the solid phase oligodeoxynucleotide synthesis but to our knowledge this kind of protection has not been used in oligoribonucleotide synthesis. The advantage of amidine protection strategy was evident since the stability studies performed under standard acidic deprotection conditions showed enhanced resistance to depurination and N,N-di-n-butylformamidine derivative of dAdo was 20 times more stable than the corresponding N^6 -benzoyl one.



	R1	R2	R3	
1	H	H	H	
2	DMTr	H	H	3
4 a	DMTr	H	tBDMSi	5 a
4 b	DMTr	tBDMSi	H	5 b
6 a	DMTr	DiPCEP	tBDMSi	7 a
6 b	DMTr	tBDMSi	DiPCEP	7 b

DMTr=4,4'-dimethoxytrityl

tBDMSi=t-butyltrimethylsilyl

DiPCEP=diisopropylamino-2-cyanoethoxy-phosphinyl

We prepared the formamidinium derivative of 8-bromoadenosine (1) using *N,N*-dibutylformamidinium dimethyl acetal and reaction conditions described for 2-deoxyadenosine. 5'-Dimethoxytritylation according to the routine procedure also occurred smoothly giving 53% of 5'- and *N*⁶-protected 8-bromoadenosine 2. As for the corresponding dAdo derivative, after 1 hr at 60 °C in 50% conc. ammonia/ethanol (v/v) containing 10% of ammonium acetate, 90% removal of dibutylformamidinium group was observed and complete deprotection was achieved in 3 hr (TLC or HPLC control, DMTr removed). The stability of the glycosidic bond in the presence of 3% dichloroacetic acid/dichloromethane was determined to be similar to that of *N*⁶-benzoyldeoxyadenosine (t_{1/2} approx. 3 hr, TLC control). These results indicated that an application of 6-*N*-[(di-*n*-butylamino)-methylene]-8-bromoadenosine (1) should be compatible with the phosphoramidite approach to the solid phase synthesis of oligoribonucleotides. For adenosine residues we decided to use a conventional *N*⁶-benzoyl protection since the benzoyl group can be completely removed under the above deprotection conditions.

The mixtures of 2'-OH and 3'-OH silylated both 5'-O-dimethoxytrityl-6-*N*-[(di-*n*-butylamino)-methylene]-8-bromoadenosine and 5'-O-dimethoxytrityl-6-*N*-benzoyl adenosine derivatives were prepared in the reaction with dimethyl-*t*-butylsilyl chloride catalyzed by imidazole. The isomers were separated by silica gel column chromatography and analyzed by ¹H and ¹³C NMR in order to assign their structures. An empirical rule to assign isomeric structure of silylated ribonucleotides has been published by Ogilvie et al. It states that silylation at a carbohydrate hydroxyl leads to a significant downfield shift of the carbon to which this hydroxyl group is attached. Unfortunately, for 5'-dimethoxytrityl protected nucleosides we observed a difference of 0.6 and 0.2 ppm (for 5a and 5b, as compared with 3) was too small to provide reliable assignment. However the same authors observed that in 5'-monomethoxytritylated silylated purine nucleosides the methyl and *t*-butyl protons of the dimethyl-*t*-butylsilyl group at the 2'-hydroxyl are significantly shifted upfield, and we based our assignment on this observation. The detailed analysis of ¹H NMR spectra of 3 and 5b also showed a downfield shift of H-3' proton (0.1 ppm) but a larger effect was exerted on the H-4' (0.2 ppm upfield). Other important changes included an upfield shift of H-2' signal and an increase in nonequivalence of protons in 5',5'' position. In 2'-silylated adenosine isomer only the H-2' signal was moved 0.3 ppm downfield.

Since no NMR data for fully protected 8-bromoadenosine have been published our assignment of structures of silylated isomers 4a and 4b was based on comparison with the corresponding adenosine derivatives. In the ¹H NMR spectrum of the isomer with larger R_f value, the H-2' proton was

deshielded (0.1 ppm), the silyl group methyl signals appeared upfield (0.2 ppm), as did the signal of H-3' (0.25 ppm). In the second isomer both the silyl group methyl signals and the H-3' proton signal were shifted significantly to lower field (0.2 and 0.4 ppm). As for 5b, there was a shielding effect on protons H-2', H-4' and H-5" (0.2, 0.1 and 0.25 ppm, respectively). Therefore for the 8-brominated isomer with larger Rf the structure of 4a was assigned, and the lower Rf isomer was identified as 4b. This assignment was later confirmed by structures of oligonucleotides synthesized from phosphoramidites 6a and 6b.

A Derivative of DDC Designed to Cross the Blood-Brain Barrier - Human immunodeficiency virus (HIV)-induced central nervous system (CNS) dysfunction, probably caused by infected monocyte/macrophage-mediated virus transfer into the brain, has been a major target of a number of studies designed to explore strategies to increase the blood-brain barrier penetration of anti-HIV agents. The nucleoside, 2',3'-dideoxycytidine, is one of the most potent dideoxynucleoside analogues *in vitro*, but it is much less effective than is AZT at penetration of the CNS based on measurements of cerebrospinal fluid concentrations.

In this investigation, we have evaluated the utility of a dihydropyridine chemical delivery system to enhance the brain uptake of 2',3'-dideoxycytidine. Specifically, we prepared a DDC derivative (HP₂DDC) which bore the 1,4-dihydro-1-methyl-3-pyridinylcarbonyl moiety at both the cytidine exocyclic amino group and the sugar 5'-hydroxyl function.

In the usual approach to the synthesis of dihydropyridinyl derivatives, a nicotinate ester is prepared which is then quaternized with methyl iodide to yield the N-methylpyridinium derivative which in turn can be reduced to the desired dihydropyridine. This approach was not feasible with 2',3'-dideoxycytidine (DDC, 1) since the cytosine ring would also be alkylated both at the ring N3 position as well as the exocyclic amino moiety. As an alternate strategy which avoided the necessity of protection of the exocyclic amino moiety of the cytosine ring, nicotinic acid methiodide was coupled to DDC (1) to give the presumed disubstituted DDC trigonellate (P₂DDC). This O- and N-acylated intermediate was not isolated but was further manipulated according to two different methodologies, depending upon the desired product. In the first approach, reduction of the diquaternized species with sodium dithionite gave the bis-dihydropyridinyl derivative (HP₂DDC, 2). Hydrolysis accomplished by overnight exposure to 0.1 M KOH in MeOH at room temperature gave the 5'-trigonellate ester of DDC (O-HPDDC, 3). The surprising preferential cleavage of the amide bond under these conditions can be rationalized by consideration of the resonance-stabilized anion that would facilitate collapse of the tetrahedral intermediate of amide hydrolysis.

In the alternative treatment, the P₂DDC intermediate was subjected to mild slightly alkaline hydrolysis at 37 °C to yield the N-acylated DDC derivative. This hydrolytic intermediate was not isolated as such, but was further treated with sodium dithionite to give N-HPDDC (4). The facile and preferential hydrolysis of the ester bond of the di-quaternized intermediate, P₂DDC, under these conditions, would be expected considering the considerable additional lability introduced to the ester bond by the proximal positively charged pyridinium group.

Chemical ionization mass spectroscopy provided a clear differentiation of the two monoacylated DDC derivatives. O-HPDDC (3) (M⁺H = 333) underwent essentially only one fragmentation to yield a peak at m/z = 222 corresponding to cleavage of the glycosidic linkage and the generation of a fragment ion consisting of the acylated sugar. This fragmentation pattern to give an ion from the acylated sugar was also witnessed in the CI-MS of N₂DDC as well as a dibenzoylated DDC. N-HPDDC (4) (M⁺H = 333) showed predominant fragmentation with loss of the N-acyl moiety to give a peak m/z 212, but significant fragmentation also occurred corresponding to loss of the sugar to give m/z 233. Thus as with other nucleosides, there is a strong tendency for glycosidic bond cleavage, but in these materials, the charge partitions to the moiety bearing the dihydropyridyl group. In the case of the CI-MS of the disubstituted HP₂DDC (2) (M⁺H = 454), both modes of fragmentation were operative to produce peaks at m/z 222 (acylated sugar) and 233 (acylated base).

To gain some idea of the ease and course of oxidation and hydrolysis of the above DDC derivatives, oxidation by DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) was examined. In addition, the

stabilities of O-HPDDC (3) and N-HPDDC (4) in Tris-acetate buffer (HPLC analysis, pH 7.4, 37 °C) were evaluated.

Generation of the requisite pyridinium derivatives was accomplished by shaking the precursors, N-HPDDC (4), O-HPDDC (3), and HP2DDC (2) with DDQ in acetonitrile followed by treatment with DEAE-Sephadex to remove the resulting red pigment. Hydrolyses of the resulting pyridinium salts were allowed to proceed in 50 mM Tris buffer (pH 7.4) at 37 °C, and aliquots were removed at various times for HPLC analysis. O-PDDC (2) was hydrolyzed to DDC (1) with 50% of the DDC being formed in 30 min. N-PDDC (4) also gave rise to DDC; however in this case, it required about 2 h for 50% of the resultant DDC to be produced. The derivative which took the greatest amount of time (3 h) to produce DDC was P2DDC. While the hydrolyses of the O-PDDC (3) and N-PDDC (4) were quite straightforward, on the other hand, the hydrolysis of P2DDC was more complicated. There was a rapid disappearance of N2DDC itself and the intermediate formation of N-PDDC which then hydrolyzed to DDC. The greater lability of the 5'-trigonellate ester bond in this situation mirrored its behavior during the synthesis of N-HPDDC.

When either O-HPDDC (3) or N-HPDDC (4) was dissolved in Tris-acetate buffer (pH 7.4, 50 mM) containing 2% acetonitrile at a concentration of 2×10^{-4} M and maintained at 37 °C, extensive decomposition occurred as monitored by HPLC. In the case of O-HPDDC (3), the half life for disappearance was approximately 7 h, but DDC (1) itself accounted for only about 30% of the product. The remaining material consisted of an unidentified byproduct. The half life for decomposition of N-HPDDC (4) was about 9 h. In this instance, about 50% of the product consisted of DDC with the remaining material being roughly equal amounts of two unidentified byproducts. It is significant that in neither situation was any O- or N-pyridinium salt detected in contrast to the oxidation studies alluded to above. When HP2DDC (2) was dissolved in the above buffer and its decomposition was followed under the same conditions, at least 15 products, including DDC (1) and N-HPDDC (4) could be detected.

To determine whether or not HP2DDC (2), O-HPDDC (3), and N-HPDDC (4) could undergo the requisite series of reactions needed to fulfill their potential as prodrug forms which could be transported into the central nervous system and be oxidized to the positively charged pyridinium form, the metabolic fates of the above congeners were examined in cytoplasmic extracts of rat brain.

At 37 °C and pH 7.4, O-HPDDC (3) was converted gradually ($t_{1/2}$ approximately 2 h) to the pyridinium form, O-PDDC, which was subsequently hydrolyzed to DDC. Control incubations in the absence of extract provided, as in previous experiments (vide supra), only small quantities of DDC with no detectable pyridinium salt. The nitrogen-substituted analogue N-HPDDC (4) behaved somewhat differently in that its $t_{1/2}$ (approximately 10 h) was considerably greater than the O-HPDDC. Also in contrast to its O-substituted isomer, N-HPDDC (4) gave rise to only low concentrations of the pyridinium form (N-PDDC), which in turn appeared to give rise to DDC. In the absence of rat brain cytosol, only minor amounts of DDC were formed.

Two conclusions could be drawn from the foregoing experiments. First, enzymic activities in the rat brain cytosol are needed to convert the dihydropyridyl prodrugs to their oxidized forms. These can then be hydrolyzed non-enzymatically to DDC as observed in the chemical oxidation experiments with DDQ. Secondly, the O-HPDDC derivative is more readily oxidized, and its pyridinium form, O-PDDC, is more readily hydrolyzed than N-HPDDC and its oxidized form N-PDDC. The more facile hydrolysis of O-PDDC as compared to N-PDDC would be expected from the ester versus amide nature of the linkages undergoing cleavage. It is not clear, however, why O-HPDDC should be more readily oxidized than N-HPDDC.

The disubstituted derivative, HP2DDC (2), was transformed by rat brain cytosol in a manner completely predictable based on the results of the experiments with the monosubstituted analogues. HP2DDC (2) itself quickly disappeared with a $t_{1/2}$ of approximately 30 min with a concurrent increase in concentration of the presumed O-P, N-HPDDC from which N-HPDDC was formed. This intermediate was then oxidized by the cytosolic enzymes to N-PDDC which then was hydrolyzed to DDC.

No evidence could be obtained from the HPLC of any formation of the disubstituted pyridinium form P₂DDC. The non-enzymatic formation of DDC was virtually insignificant under these conditions.

When evaluated for their ability to inhibit the growth of human immunodeficiency virus (HIV-1) in human lymphocyte MT-4 cells, O-HPDDC and N-HPDDC each gave IC₅₀ values of 1.2×10^{-6} M, HP₂DDC showed an IC₅₀ of 1.9×10^{-6} M, and DDC as reference standard had an IC₅₀ of 1.0×10^{-6} M. These results suggested that all three dihydropyridyl derivatives could be transformed to DDC by cell cultures and were therefore virtually equivalent to DDC in their antiviral potential.

We followed the time-dependent plasma and brain concentrations of DDC (1) following its i.v. administration to rats (23 mg/kg). A peak plasma level of 115.8 ± 3.3 nmol/ml occurred at 5 min, the earliest measured time, and thereafter DDC concentrations declined with a half-life of 22.7 min. No drug was detected after 240 min. Dramatically lower levels were present in brain, with a peak concentration of 0.44 ± 0.16 nmol/g occurring at 120 min. The PA of DDC was 3.4×10^{-6} s⁻¹, and its brain/plasma concentration integral ratio was 0.04.

Also we examined the time-dependent concentrations of HP₂DDC (2), DDC (1) and combined (total) levels in plasma and brain, respectively, following i.v. administration of HP₂DDC (49.3 mg/kg). A peak level of 36.6 ± 7.3 nmol/ml HP₂DDC (2) was detected in plasma at 5 min, which declined to 0.3 ± 0.1 nmol/ml at 480 min. Low concentrations of DDC were detected up to 240 min, with a peak level of 2.0 ± 0.6 nmol/ml occurring at 5 min. HP₂DDC was detected in brain throughout the study, reaching a peak concentration of 7.7 ± 2.9 nmol/g at 15 min. Low levels of DDC also were detected, with a peak concentration of 1.4 ± 0.5 nmol/g at 240 min. The brain/plasma concentration integral ratio of HP₂DDC was 0.95, whereas that for DDC in brain as a ratio of combined HP₂DDC and HP₂DDC levels in plasma was 0.24.

We investigated the time-dependent concentrations of N₂DDC (5), DDC and combined (total) levels in plasma and brain, respectively, after i.v. administration of N₂DDC (46 mg/kg). A peak concentration of 54.0 ± 2.1 nmol/ml N₂DDC (5) was detected in plasma at 5 min, declining to 1.9 ± 1.0 nmol/ml at 480 min. DDC was present in plasma between 5 and 120 min, with a peak level of 13.1 ± 2.2 nmol/ml at 5 min. Low concentrations of both N₂DDC (5) and DDC (1) were detected in brain throughout the study, with peak levels of 1.2 ± 0.4 nmol/g at 30 min and of 0.9 ± 0.7 nmol/g at 5 min, respectively. The brain/plasma concentration integral ratio of N₂DDC was 0.12, whereas that for DDC in brain as a ratio of total agents in plasma was 0.04. The disappearances of DDC, HP₂DDC and N₂DDC from plasma were similar, although a higher peak concentration was achieved after DDC injection. Furthermore, brain concentrations of DDC were low compared to total drug in plasma for each agent. Although HP₂DDC maintained the highest concentration in brain and achieved the largest time-dependent concentration integral, these were not significantly different from those achieved following equimolar administration of DDC or N₂DDC.

Earlier studies have concluded that, in the case of the application of the dihydropyridine-pyridinium redox chemical delivery system to the anti-HIV agent AZT, a significant increase in brain exposure to the antiviral agent was possible. In our attempts to apply this redox drug delivery system to DDC which is substantially less effective than is AZT at crossing the blood-brain barrier, we have not been able to demonstrate a significant increase in brain exposure to DDC after administration of the disubstituted redox prodrug, HP₂DDC (2). We have found this kind of DDC derivative relatively difficult to access synthetically as compared to the corresponding AZT derivative. The lipophilic nature of these materials represents a significant experimental difficulty which would surely complicate therapeutic application. For reasons we do not understand, the series of DDC derivatives (HP₂DDC, O-HPDDC, and N-HPDDC) were significantly less stable under mild conditions than was the corresponding AZT congener with which we have also worked. This instability also complicates experimental manipulation of these redox prodrug forms of DDC and, of course, would also be a therapeutic liability. It is possible that this liability may at least partly explain the failure of the application of this particular prodrug approach to achieving greater brain exposure to DDC.

Uronic Acid Analogues of 2-5A: Synthesis and Biological Activity - The oligonucleotide ppp5'A2'p5'A2'p5'A, known as 2-5A, is a potent translational inhibitor involved in some aspects of interferon action. To explore the specific function of the charged 5'-triphosphate moiety, we prepared a series of congeners in which the 5' region was hypermodified. Thus, uronic acid derivatives were substituted for the 5' terminal adenosine residue of 2-5A. Compounds 9, 10, 11, and 12 carried adenosine 5'-uronic acid, ethyl adenosine 5'-uronate, adenosine 5'-uronamide, and adenosine 5'-(n-ethyl)uronamide, respectively, in place of the 5' terminal adenosine triphosphate moiety of 2-5A. While all the analogues showed some weak interaction with the 2-5A-dependent endonuclease (RNase L), compound 9 showed the strongest binding ability, and while unable to activate the mouse RNase L, could activate human RNase at a concentration 100-fold greater than that required for the parent 2-5A. This result suggests that the function of the 5'-(poly)phosphate moiety of 2-5A may be fulfilled by some other anionic moiety.

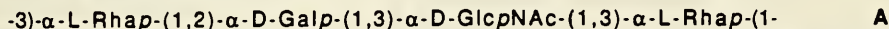
New Approach to the Synthesis of 8-Bromoadenosine Analogues of 2'5'-Oligoadenylates -

A solid phase oligonucleotide synthetic method was developed to provide easier access to 8-bromoadenylate-2',5'-linked oligonucleotides which show activity as activators of the 2-5A-dependent ribonuclease. The standard CPG-LCA solid support and the phosphoramidite approach could be employed, but while N⁶-benzoyl protection was satisfactory for non-modified adenine residues, 8-bromoadenosine required non-acyl protection. Specifically, the N,N-di-n-butylformamidine derivative of deoxyadenosine was found to be the most useful. A variety of mixed sequence adenosine/8-bromoadenosine oligonucleotides could be synthesized using this modified technology.

A New and Potent 2-5A Analogue Which Does Not Require a 5'-Polyphosphate to Activate Mouse L Cell RNase L - In order to explore the possibility of supplanting the requirement of a 5'-triphosphate moiety for the activation of the 2-5A-dependent endonuclease (RNase L) of mouse L cells, two new tetrameric analogues of 2-5A were synthesized. The first tetramer, obtained by both a modified prebiotic synthetic approach as well as a phosphite triester solid phase oligonucleotide synthesis method, was p5'A2'p5'A2'p5'(br8A)2'p5'(br8A). The second oligonucleotide was derived from the former by a sequence involving periodate oxidation, reaction with n-hexylamine, and cyanoborohydride reduction, resulting in conversion of the 2'-terminal adenosine residue to 9-(3'-aza-4'-hexyl-1',2',3',4'-tetrahydroxyhexopyranos-1'-yl)-8-bromoadenine. Both of these oligomers were found to be as potent as 2-5A itself as activators of the RNase L of mouse L cells.

SECTION ON CARBOHYDRATES

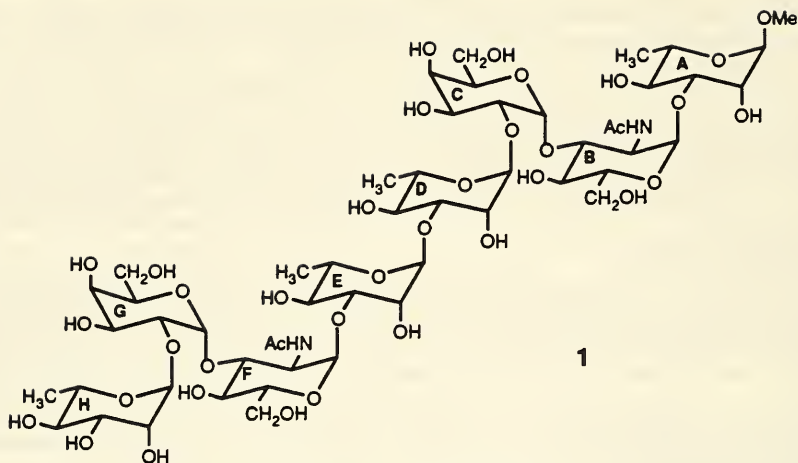
Shigella dysenteriae type 1 is a human pathogen causing dysentery with a high incidence of mortality. The virulence of this pathogen requires full expression of its lipopolysaccharide (LPS): indirect evidence suggests that the O-specific polysaccharide (O-SP) is also a protective antigen in humans. Its LPS contains tetrasaccharide A as the repeating unit of the O-SP region. It is an attractive assumption that protective, anti O-



SP antibodies might also be elicited by using smaller fragments of the native, O-SP. Oligosaccharides (OSs) that mimic the properties of the native O-SP define the minimum structural requirements of such immunodeterminants. We are studying the synthesis of OSs possessing extended chain-length related to A.

As part of this project, we describe a synthetic route to octasaccharide methyl glycoside **1**, which corresponds to two contiguous repeating units of the O-SP of the LPS of *S. dysenteriae* type 1.

The synthesis of octasaccharide **1** is based on a [4 + 4] block approach: two tetrasaccharides (**36** and **41**) were prepared, corresponding to units A-D⁶ and E-H⁶ and were combined to provide a



1

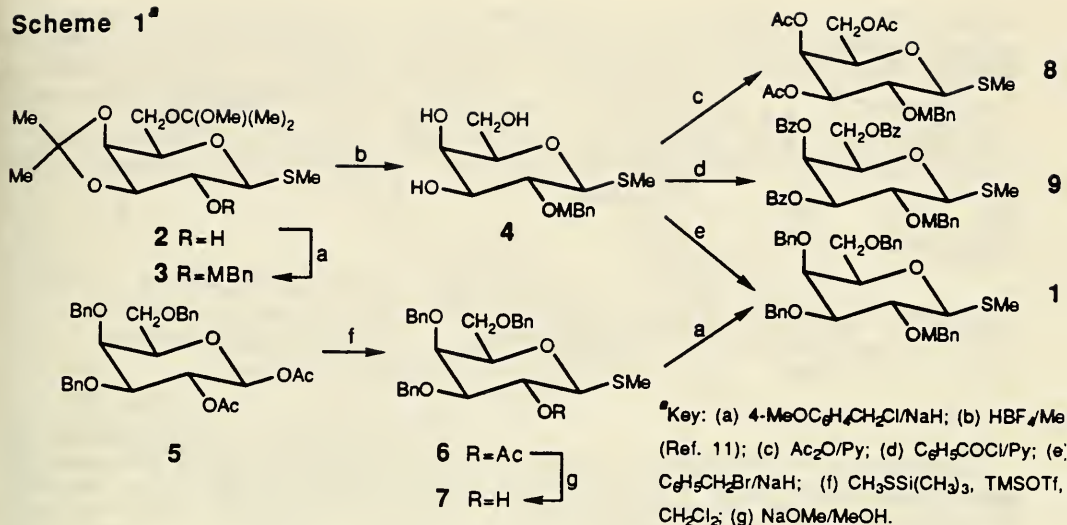
protected derivative of 1. Tetrasaccharide acceptor 36, and donor 41 were assembled in a stepwise manner using heterofunctional D-galacto-, D-gluco-, and L-rhamnopyranoside derivatives, the syntheses of which are described next.

D-Galactose synthons (Scheme 1). Thioglycosides 8-10 were selected as galactosyl donors. Their common feature is the non-participating, 4-methoxybenzyl group at *O*-2. This type of protection allows the stereoselective formation of a 1,2-*cis* interglycosidic linkage upon activation of the anomeric center by thiophilic reagents. The sensitivity of the 4-methoxybenzyl group to oxidation permits selective unmasking in the presence of a variety of protective groups which is a favourite feature for a multifunctional oligosaccharide intermediate. Triol 4 (Scheme 1) was obtained from mixed acetal 2 in two steps using the fully protected thiogalactoside 3 (64%) and converted directly to acyl- [8 (92%) and 9 (90%)] and benzyl-protected thiogalactoside donors [10 (93%)] in conventional reactions. An alternative route to 10 utilized diacetate 5 which was converted to thioglycoside 6 (82%) ($\text{CH}_3\text{SSi}(\text{CH}_3)_3$, TMSOTf, CH_2Cl_2) followed by deacetylation to give alcohol 7 (83%) which was then 4-methoxybenzylated at *HO*-2 to provide 10 (85%).

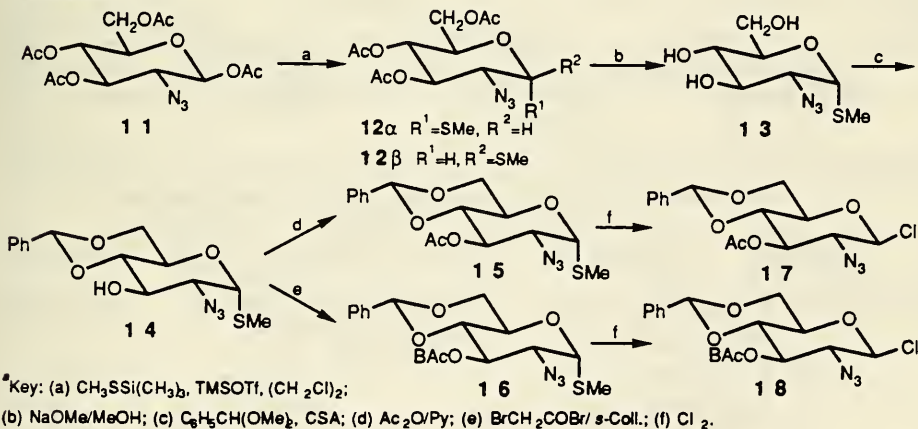
N-Acetyl-D-glucosamine synthons (Scheme 2). Tetraacetate 11 was the starting material for the glucosamine synthons 15-18. It was converted to thioglycoside 12 ($\text{CH}_3\text{SSi}(\text{CH}_3)_3$, TMSOTf, CH_2Cl_2) (93%, 12 α /12 β 4:1). Routine transformation of 12 α by way of alcohols 13 and 14 [(i) de-*O*-acetylation, (ii) benzylidene acetal-formation, (iii) acylation] provided acetate 15 and bromoacetate 16 respectively; these can be used as donors, either directly, or after conversion to crystalline glycosyl chlorides 17 and 18.

D-Rhamnose synthons (Scheme 3). The intermediate for residue D was rhamnosyl donor 28, first prepared by Pavliak *et al.* Its 2-*O*-benzoyl group anchimerically assists the stereocontrolled formation of a 1,2-*trans* interglycosidic linkage, and the bromoacetyl group permits selective unmasking of *HO*-3 without compromising the other linkages. Compounds 19 and 20 were first converted to intermediate, cyclic orthoesters 21 and 22, which were then benzoylated at *HO*-4. Regioselective opening of the orthoester ring with acetic acid provided the 2,4-dibenzoates 23 and 24 [69-88% yields for three steps (a-c)]. Subsequent bromoacetylation at *HO*-3 yielded the fully protected rhamnosides 25 and 26. Thioglycoside 25, and 2-(trimethylsilyl)ethyl glycoside 26, were converted to chloride 28 directly by chlorine and α,α -dichloromethyl methyl ether (DCMME)/ ZnCl_2 respectively. Alternatively, chlorination (DCMME/ ZnCl_2) of acetate 27 provided rhamnosyl donor 28 in 98% yield.

Scheme 1^a

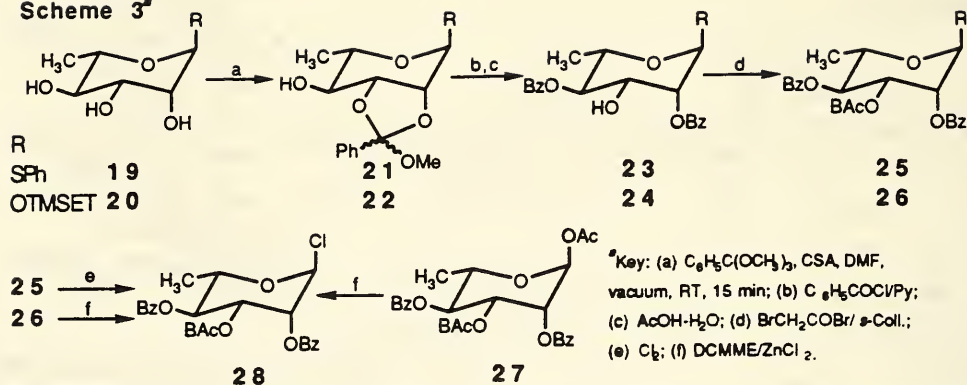


Scheme 2^a



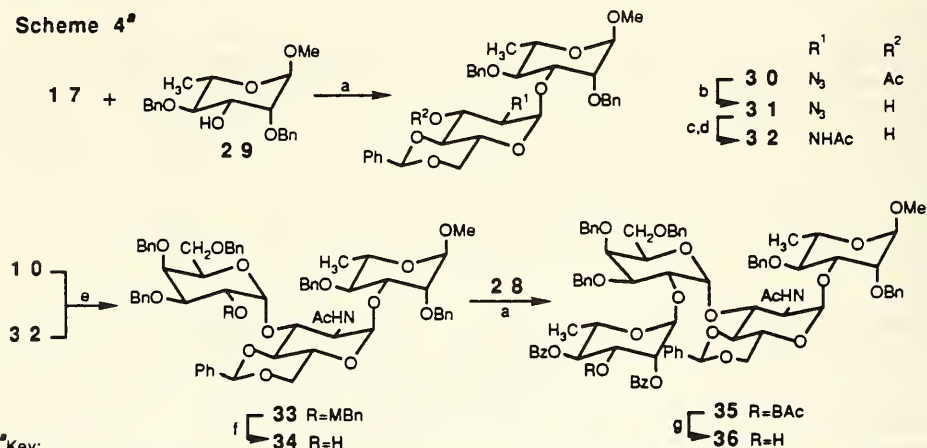
Stereoselective coupling of glucosamine donor **17** with the previously described alcohol **29** (AgOTf) afforded disaccharide **30** in 65% yield (Scheme 4). Removal of the acetyl group (NaOMe) gave alcohol **31** which was converted into acceptor **32** by azide-reduction (NiCl₂/H₃BO₃) followed by *N*-acetylation (79%). Glycosylation of **32** with **10** (MeOTf, ether) provided trisaccharide **33** (87%) which was treated with DDQ to provide alcohol **34** (57%). Reaction of rhamnosyl chloride **28** with nucleophile **34** (AgOTf) gave fully protected tetrasaccharide **35** (94%) which was debromoacetylated to provide the acceptor **36** (94%).

Scheme 3^a

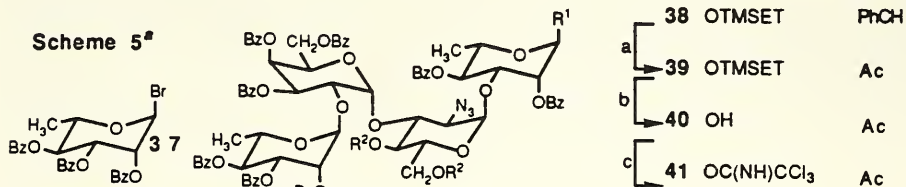


Compounds **9**, **18**, **24**, and **37** were used for the *stepwise* synthesis of tetrasaccharide **36** employing conditions for glycosylation and deprotection similar to those used for the synthesis of **36** (61% yield for five steps). Sequential replacement of the benzylidene by acetyl groups (**38**→**39**), hydrolytic removal of the trimethylsilylethyl group (**39**→**40**) and imideate formation afforded tetrasaccharide donor **41** (75% for three steps).

Scheme 4^a

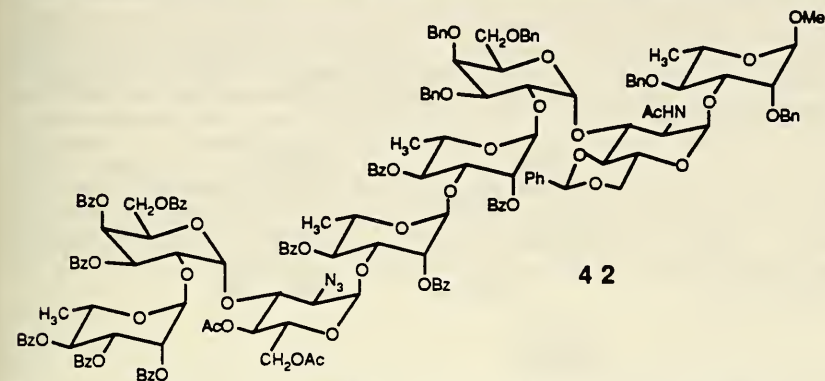


Scheme 5^a



Combination of tetrasaccharide acceptor **36** and donor **41** (BF₃.Et₂O) gave fully protected octasaccharide **42** (69%). Its routine transformation by azide reduction (NiCl₂/H₃BO₃), N-acetylation (Ac₂O), de-O-acylation (NaOMe) and debenzoylation (H₂/Pd-C), gave the free octasaccharide methyl glycoside **1**.

The chemical shifts of the anomeric protons in the O-specific PS **A**, octasaccharide **1**, hexasaccharide **43**, pentasaccharide **44**, and tetrasaccharides **45**, **46** (Table) reveal a dependence of



- 43** α-L-Rhap-(1,3)-α-L-Rhap-(1,2)-α-D-Galp-(1,3)-α-D-GlcpNAc-(1,3)-α-L-Rhap-(1,3)-α-L-Rhap-OMe
44 α-L-Rhap-(1,3)-α-L-Rhap-(1,2)-α-D-Galp-(1,3)-α-D-GlcpNAc-(1,3)-α-L-Rhap-OMe
45 α-L-Rhap-(1,2)-α-D-Galp-(1,3)-α-D-GlcpNAc-(1,3)-α-L-Rhap-OMe
46 α-D-Galp-(1,3)-α-D-GlcpNAc-(1,3)-α-L-Rhap-(1,3)-α-L-Rhap-OMe

Table 1 ¹H-NMR chemical shifts (ppm) of the anomeric protons of the O-specific polysaccharide of *Shigella dysenteriae* type 1 and oligosaccharides **1**, and **43-46**^a

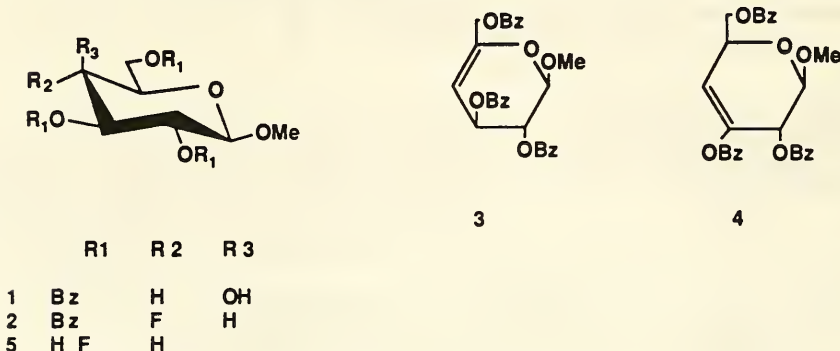
Compound	Chemical shift ^b							
	Rha	Gal	GlcN	Rha	Rha	Gal	GlcNRha	Rha
O-SP (A)	5.11	5.60	5.04	5.11	5.05	5.60	5.04	5.11 5.05
1	5.08	5.60	5.04	5.11	5.05	5.60	4.99	4.71
43				5.08	5.05	5.60	5.04	5.05 4.66
44				5.07	5.05	5.60	4.99	4.71
45					5.09	5.61	5.00	4.73
46						5.42	5.04	5.07 4.66

^aIn D₂O at 296K, int. acetone δ=2.225 ppm. ^bChemical shifts for the anomeric protons of the O-SP and for those in the oligosaccharides which coincide with the corresponding resonances of the O-SP are shown in **boldface**.

these parameters on the length of the OS. The chemical shift of only one anomeric proton in each of compounds **45** and **46** is identical with that of the corresponding proton in the O-SP. That number of common resonances is two for the O-SP/pentasaccharide **44** and three only for the O-SP/hexasaccharide **43** i.e. none of the OSs **43-46** embodies a *complete* repeating unit sequence of four residues whose conformation mimics that of the corresponding fragment in the O-SP. In octasaccharide **1**, the chemical shifts of the anomeric protons of five consecutive residues coincide with the corresponding resonances of the O-SP. It is probable that in **1** the fragment consisting of residues C-G

resembles the conformation of the O-SP. Thus octasaccharide 1 is expected to be a valuable hapten for the preparation of synthetic antigens, which is in progress in these laboratories.

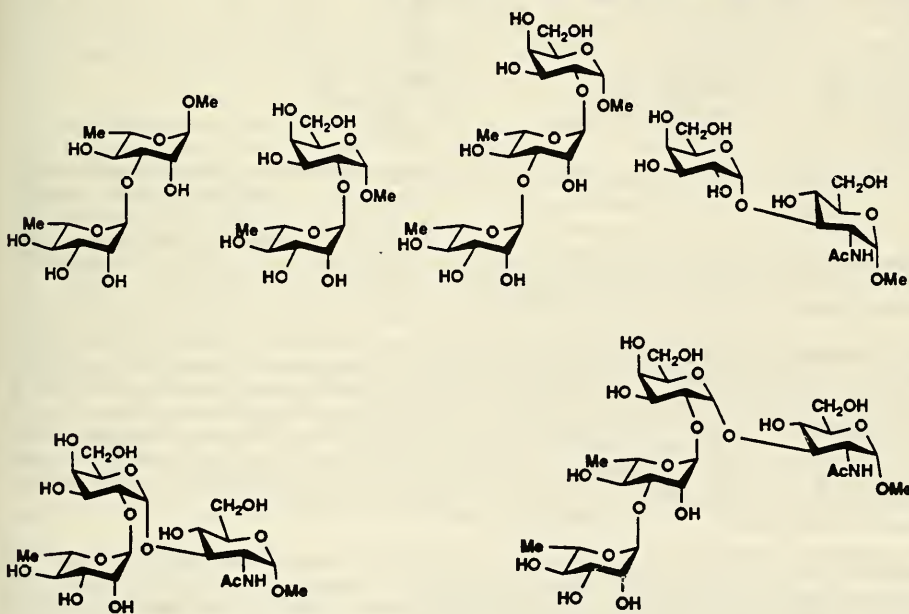
In the past, we have used deoxyfluoro sugars as probes for studies of ligand-antibody interactions. Investigations of the putative role of hydrogen bonding in the binding process involving antibodies that show specificity for epitopes containing β -D-glucopyranose required hitherto unknown methyl 4-deoxy-4-fluoro- β -D-glucopyranoside (5). For the synthesis of 5, we have chosen the readily available methyl 2,3,6-tri-O-benzoyl- β -D-galactopyranoside (1) as the starting material, and diethylaminosulfur trifluoride (DAST) as the fluorinating reagent. Although DAST has been frequently used to produce fluorinated carbohydrates in high yields, the outcome of the reaction is somewhat unpredictable. We believe what we found is unique in that two products of elimination have been formed, as a consequence of attacks of the proton-abstracting species at different sites in the molecule.



Initially, compound 1 was treated with DAST (100% molar excess) in 1,2-dimethoxyethane at room and elevated temperatures in the absence of a base. In these reactions the conversion of 1 was either largely incomplete, or the reaction mixtures contained a large proportion of by-products (60 °C). An appreciable amount of undesired products was still formed when the reaction was carried out in the presence of pyridine at 60 °C but, in this solvent, the number and the overall amount of by-products formed was smaller than when the reaction was conducted in the absence of the base. The three major products of such a reaction were isolated by chromatography, identified and fully characterized. The expected methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-fluoro- β -D-glucopyranoside (2, showing the slowest chromatographic mobility) was isolated in 47.7% yield. The amounts of the isolated olefinic by-products 3 (showing fastest chromatographic mobility) and 4 were 23.4 and 12.8%, respectively. Thus the remainder of the reaction products accounting for less than some 10%, consisted of several trace impurities, and were not further investigated. Conventional debenzoylation of 2 readily yielded the crystalline target compound 5. When the reaction was conducted in dichloromethane as the solvent, in the absence of a base, the formation of the olefinic by-products was somewhat less pronounced (TLC), similar to what has been found by others. The structures 2 and 5 followed clearly from the first-order analysis of the one-dimensional ^1H - and ^{13}C -NMR spectra, and the structures 3 and 4 were deduced, *inter alia*, from the analysis of 2D NMR spectral data. The olefinic nature of these compounds was supported by the ^{13}C -NMR spectra showing signals in the region characteristic of unsaturated carbons. The comparable chemical shifts observed for the methylene protons H-6a and H-6b in 1-4 suggest that the olefinic by-products do not contain an exocyclic double bond. Rather, the observed chemical shifts, δ 150.69 in the spectrum of 3 and δ 142.61 in the spectrum of 4, assigned to C-5 and C-3, respectively, are consistent with these carbons being part of an enolic arrangement.

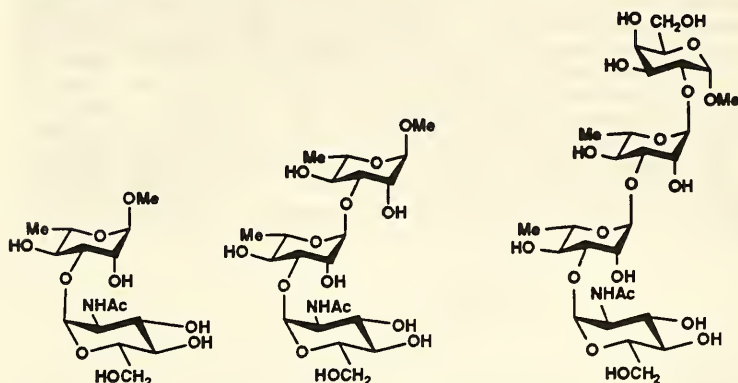
The unambiguous assignment of lines in the NMR spectra of 3 followed from results of 2D NMR experiments. Unsaturated products 3 and 4 are formed by common elimination that frequently accompanies $\text{S}_{\text{N}}2$ substitution reactions during fluorinations with DAST or with other nucleophiles. Their formation in an approximate ratio of 2 : 1 (see Experimental) quite likely reflects the more acidic nature of H-5 in 1, as compared with H-3, due to the proximity of the ring oxygen atom.

Methyl *O*-(2,3,4-tri-*O*-benzoyl- α -L-rhamnopyranosyl)-(1 \rightarrow 3)-2,4-di-*O*-benzoyl- α -L-rhamnopyranoside, obtained by silver trifluoromethanesulfonate-mediated condensation of methyl 2,4-di-*O*-benzoyl- α -L-rhamnopyranoside and 2,3,4-tri-*O*-benzoyl- α -L-rhamnopyranosyl bromide (3), was cleaved with dichloromethyl methyl ether (DCMME) to give *O*-(2,3,4-tri-*O*-benzoyl- α -L-rhamnopyranosyl)-(1 \rightarrow 3)-2,4-di-*O*-benzoyl- α -L-rhamnopyranosyl chloride (9). Condensations of 1,3,4,6-tetra-*O*-acetyl- α -D-galactopyranose with 3 and 9, followed by treatment of the products with DCMME yielded, respectively, glycosyl chlorides 12 and 17. Each of these, as well as 2,3,4,6-tetra-*O*-benzyl- α -D-galactopyranosyl chloride was condensed with 4,6-*O*-substituted (benzylidene, tetraisopropylidisiloxane-1,3-diyl, or benzyl) derivatives of methyl 2-acetamido-2-deoxy- α -D-glucopyranoside, using CH_2Cl_2 , ether, or a mixture thereof as the solvent. The formation of the desired α -D-galactopyranosyl linkage was favored when ether was the solvent. Under these conditions, however, the combined yield of the condensation products decreased, especially when less reactive synthons were used. The α -linked products obtained were deprotected to give the methyl α -glycosides of the tetra, tri-, and the disaccharide related to the chemical repeating unit of the *O*-specific side chain of the lipopolysaccharide of *Shigella dysenteriae* type 1. Synthesis of methyl α -glycosides of three other constituents of the same polymeric antigen were also prepared. All together, the following methyl α -glycosides of oligosaccharides related to the chemical repeating unit of the *O*-specific antigenic polysaccharide of *Shigella dysenteriae* type 1, namely $\rightarrow[3-\alpha\text{-L-Rhap-(1\rightarrow3)-}\alpha\text{-L-Rhap-(1\rightarrow2)-}\alpha\text{-D-Galp-(1\rightarrow3)-}\alpha\text{-D-GlcpNAc-1}]_n\rightarrow$ have been synthesized:



Stereoselective Syntheses of a Di-, Tri-, and a Tetrasaccharide Fragment of *Shigella Dysenteriae* Type 1 *O*-Antigen Using 3,4,6-Tri-*O*-Acetyl-2-Azido-2-Deoxy- α -D-Glucopyranosyl Chloride as a Glycosyl Donor - Methyl 2,4-di-*O*-benzoyl- α -L-rhamnopyranoside (1) was *O*-bromoacetylated and the resulting, crystalline 3-*O*-bromoacetyl derivative was treated with dichloromethyl methyl ether- ZnCl_2 reagent to give 2,4-di-*O*-benzoyl-3-*O*-bromoacetyl- α -L-rhamnopyranosyl chloride (2). Compounds 1 and 2 were condensed under the conditions of base-deficient silver trifluoromethanesulfonate-mediated glycosylation, to give the fully protected rhamnobioside 3 which was de-*O*-bromoacetylated, affording the disaccharide nucleophile 4. Similar condensation of 2 with methyl 3-*O*-benzoyl-4,6-*O*-benzylidene- α -D-galactopyranoside, followed by de-*O*-

bromoacetylation, and condensation of the thus formed methyl O-(2,4-di-O-benzoyl- α -L-rhamnopyranosyl)-(1 \rightarrow 2)-4,6-O-benzylidene-3-O-benzoyl- α -D-galactopyranoside with **2** gave the corresponding trisaccharide. Subsequent de-O-bromoacetylation gave **5** having only HO-3 unsubstituted. Silver perchlorate-mediated glycosylations of each of **1**, **4** and **5** with 3,4,6-tri-O-acetyl-2-azido-2-deoxy- α -D-glucopyranosyl chloride afforded, with high stereoselectivity, the corresponding di, tri-, and a tetrasaccharide, all containing 2-azido-2-deoxy- α -D-glucopyranosyl end-group. Subsequent hydrogenation, followed by N-acetylation, and de-O-acylation afforded the following three oligosaccharides that make up parts of the repeating unit of *Shigella dysenteriae* type 1 O-antigen:



A series of synthetic, fully acetylated methyl monodeoxy-monofluoro- α -D-glucopyranosides have been studied using electron impact and ammonia chemical ionization mass spectrometry. Mass analyzed metastable ion kinetic energy (MIKE) spectroscopy, collision activation (CI), and accelerated voltage scanning have been used to evaluate complete fragmentation schemes. Characteristic differences in the fragmentation of positional isomers were noted on analysis of the spectra, and these make it possible to determine the location of fluorine in hexopyranosides.

Sequence analysis of oligosaccharides is most frequently based on spectra obtained from derivatized compounds. The fast-atom bombardment (FAB) mass spectra obtained from a native sample primarily contain molecular mass information in the form of one or more pseudomolecular ions. The intensities of the fragment ions are often very low in comparison to the signals of matrix or impurities. For this reason, carbohydrates are usually derivatized prior to obtaining FAB spectra. To assist in the determination of the structure of oligosaccharides without derivatization we measured FAB spectra using various matrices and B/E linked scans with or without the presence of helium as the collision gas, to enhance fragmentation in the first field-free region of the instrument.

As model substances we used synthetic methyl β -glycosides of D-galacto-oligosaccharides, some of which were fluorinated or deoxygenated at certain positions. The samples were dissolved in water, mixed with various matrices on the target, and subjected to xenon (6 kV) bombardment. The method based on the B/E linked measurement of FAB mass spectra, obtained on the m-bullet matrix, was found to be most successful. The B/E linked FAB spectra contain intense "sequence ions" allowing sequence analysis of oligosaccharides, while the fragment ions derived from the matrix are not present. This is very much unlike the case of conventional FAB spectra, where the structurally significant ions are obscured by fragment ions derived from matrix molecules.

The partial elucidation of the binding mode of an anti-*Shigella dysenteriae* type 1 murine monoclonal IgM toward its antigenic determinant is proposed. This is based on the affinities of the pentameric IgM for eighteen synthetic fragments of the O-polysaccharide, and on the affinity of the IgM-Fab for the intact O-specific bacterial polysaccharide. The results show that the galactosyl residue is the only one of the monosaccharides present in the antigenic epitope that by itself shows quantifiable binding. It is contributing from 2.7 to 3.0 kcal/mol of binding free energy, depending on

the structure of the synthetic ligand-fragment it is a part of. Addition of one rhamnosyl residue, glycosidically linked $\alpha(1\rightarrow2)$ to the galactosyl unit, increases the free binding energy by 1.4 to 2.2 kcal/mol, depending again on the particular ligand. Protein binding subsites for these two sugars are called subsite A and B, respectively. Further extension of the oligosaccharidic ligand toward the "non-reducing" end (glyconic terminus) by rhamnosyl and glucosacetaminyl moieties improves the binding only minimally. The maximal association constant ($K_a = 9.5 \times 10^3 \text{ M}^{-1}$, $-\Delta G = 5.4 \text{ kcal}$) is for the tetrasaccharide determinant $\alpha\text{-D-GlcpNAc}-(1\rightarrow3)\text{-}\alpha\text{-L-Rhap}-(1\rightarrow3)\text{-}\alpha\text{-L-Rhap}-(1\rightarrow2)\text{-}\alpha\text{-D-Galp-OMe}$. That affinity constant is however nearly identical to that of the disaccharide $\alpha\text{-L-Rhap}-(1\rightarrow2)\text{-}\alpha\text{-D-Galp-OMe}$ ($K_a = 6.5 \times 10^3 \text{ M}^{-1}$, $-\Delta G = 5.3 \text{ kcal}$). Extension of the oligosaccharidic chain toward the "reducing" end (aglyconic terminus) decreases the binding energy by 0.3 to 1.1 kcal, depending on the structure of the synthetic ligand. This suggests that small conformational effects might be involved. Affinity measurements using IgM-Fab and the intact O-specific polysaccharide show that the antibody is capable of binding internal segments on the antigen chain. The unusually low association constant ($K_a = 2.7 \times 10^3 \text{ M}^{-1}$) of this antibody, might define the lower limit for an immune response to an immunogenic epitope.

Finally, our subsite studies on monoclonal anti-galactan were concluded by binding studies on determinants flanked by non-binding sequences. The penta- through octasaccharides $\beta\text{-D-Glcp}-(1\rightarrow6)\text{-}\beta\text{-D-Glcp}-(1\rightarrow6)\text{-}[\beta\text{-D-Galp}-(1\rightarrow6)]_n\text{-}\beta\text{-D-Glcp}-(1\rightarrow6)\text{-}\beta\text{-D-Glcp-1}\rightarrow\text{OMe}$, ($n=1-4$) were prepared by a convergent block-synthesis. The haloacetyl-, tert-butyldiphenylsilyl and the dimethylhexylsilyl group, respectively were used as temporary protective groups for the preparation of the intermediate glycosyl donor and acceptor building blocks. The deoxygenated trisaccharides $\beta\text{-D-Glcp}-(1\rightarrow6)\text{-}\beta\text{-D-Galp}-(1\rightarrow6)\text{-}\beta\text{-4-deoxy-D-xylo-Hexp-1}\rightarrow\text{OMe}$ and $\beta\text{-D-Glcp}-(1\rightarrow6)\text{-}\beta\text{-4-deoxy-D-xylo-Hexp}-(1\rightarrow6)\text{-}\beta\text{-D-Galp-1}\rightarrow\text{OMe}$, respectively were synthesized accordingly. All oligosaccharides were used to study their binding to monoclonal antigalactan antibody IgA J539. The results strongly support the previous finding that J539 can bind to internal antigenic epitopes and that subsite C of that antibody binds glucose with a K_a of about 6 (in contrast to galactose which is bound to subsite C with a K_a of 10.9).

1990-1991 Non-project Activity.

Dr. Kenner C. Rice (Chief, LMC) continues as a member of the Editorial Advisory Board of the *Journal of Medicinal Chemistry* and has completed his three year term as a member of the Executive Committee of the Organic Chemistry Division of the American Chemical Society (ACS). In the latter capacity, he served as liaison with the Biotechnology Secretariat of the ACS. He also completed a four year term of service on the Board of Directors of the College on Problems of Drug Dependence (CPDD), and he has been elected a Charter Fellow of the CPDD. Dr. Rice served as a member of Peer Review Panel C for the Walter Reed Army Institute of Research Promotion Board. He has been appointed to the Advisory Board of *Medicinal Chemistry Reviews*. During the reporting period, Dr. Rice has been invited to present a number of research lectures in the U.S. and abroad. Dr. Rice was reappointed as Adjunct Professor of Pharmacology in the Department of Pharmacol. and Exp. Therap., School of Medicine, UMBC. He continues to serve on the selection committee for national and international research awards.

Dr. Arthur E. Jacobson (Deputy Chief, LMC) was reappointed as Biological Coordinator of the Drug Evaluation Committee of the College on Problems of Drug Dependence (CPDD) for 1991-1992, and as Affiliate Professor in the Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University. He is a member of the Editorial Board of the journal *Drug and Alcohol Dependence*. He continues to serve on the selection committee for the international Sato Memorial award, and is on the Research Evaluation Panel for the Walter Reed Army Institute of Research. Dr. Jacobson has been elected a Charter Fellow of the CPDD.

Patent Applications. In the Drug Design and Synthesis Section one new patent was filed this year and 13 other patents are pending. They include (a) Nitrogen-containing cycloheterocycloalkyl aminoaryl derivatives for CNS disorders; (b) N-(arylethyl)-N-alkyl-2-(1-pyrrolidinyl) ethylamines, a novel class of neuroprotective sigma receptor ligands; (c) radiolabeled N-substituted-6-iodo-3,14-dihydroxy-4,5- α -epoxymorphinans, intermediates for producing the same, and a process for the preparation and methods of detecting opioid receptors; and, (d) (+)-isomers of endoetheno/endoethano-epoxymorphinan derivatives as antitussive agents.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 59,501-06 LMC

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Design and Synthesis of Drugs Acting on Central and Peripheral Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K. C. Rice

Chief, Drug Design & Synth. Section

LMC-NIDDK

Co PI: A. E. Jacobson

Deputy Chief, LMC

LMC-NIDDK

Others:

W. Bowen, Staff Fellow; B.R. de Costa, Visiting Associate; Z. Gu, NRC Fellow; N.A. Grayson, C. Dominguez, IRTA; W. Williams, Microbiologist; H. Xu, Visiting Associate; L. Radesca, Special Volunteer; J.T.M. Linders, D. Tadic, S. He, D. Matecka, S. Calderone, Visiting Fellow. All LMC, NIDDK.

COOPERATING UNITS (if any)

LN-NIDDK (P. Skolnick); U Arizona (F. Porreca); U of Michigan (J. Woods, C.P. France); CC-NM (D. Kiesewetter, M. Channing); U Alabama (E. Blalock); BPB-NIMH (A. Pert); Research Triangle Institute (F.I. Carroll); ARC, NIDA (J.L. Katz, R.B. Rothman, H.C. Akunne); NCI (T.R. Burke Jr.).

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

Drug Design and Synthesis Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

9.5

PROFESSIONAL:

8.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Synthetic programs are continuing to develop new ligands for imaging brain drug receptors by positron emission tomography (PET) and single photon emission computed tomography (SPECT) scanning, and the NIH Opiate Total Synthesis is employed to provide previously inaccessible unnatural enantiomers of opiates and derivatives. The binding characteristics, pharmacology, immunochemistry, and the multiplicity of opioid receptors were examined, and new drugs were explored as treatment agents for cocaine abuse and for their interaction with the dopamine transporter.

Multiple delta opioid receptors. Ligand binding data, utilized to determine whether subtypes of the delta opioid receptor existed in rat brain membranes, supported pharmacological studies demonstrating delta receptor subtypes which mediate antinociception.

Immunoregulatory opioids - Opioid compounds, such as morphine and beta-endorphin, are active immunoregulatory molecules in vitro and in vivo, and opioid receptors are present on immune cells. We found that the lymphocyte mu-class binding site has a molecular weight of 58 kDa under nonreducing conditions and 70 kDa under reducing conditions, compared with brain mu-class binding sites which were found to have a molecular weight of 54 kDa under nonreducing conditions. These and other data indicate the presence of a functional m-type opioid receptor on cells of the immune system and add to the concept of bidirectional circuitry between the immune and neuroendocrine systems.

Potential treatment agents for cocaine abuse - The high affinity dopamine reuptake inhibitor 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine (GBR12909) produced a dose-dependent decrease in the binding of [3H]cocaine or [3H]GBR12935 to the dopamine transporter. GBR12909 antagonizes the ability of cocaine to elevate extracellular dopamine by 50%. Further studies will be needed to evaluate a possible role for GBR12909 in the medical treatment of cocaine addiction.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 59,502- 06LMC

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Design, Synthesis and Evaluation of Medicinal Agents and Research Tools

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A.E. Jacobson	Deputy Chief, LMC	LMC-NIDDK
Co PI: K.C. Rice	Chief, Drug Design & Synth. Sect.	LMC-NIDDK
Others: B.R. de Costa, H. Xu	Visiting Associate	LMC-NIDDK
M. Mattson, J.M. Cutts, C. Dominguez	Biologist, IRTA, IRTA	LMC-NIDDK
J.T.M. Linders, S. He	Visiting Fellow	LMC-NIDDK
W. Bowen, B. Vilner	Staff Fellow, Expert	LMC-NIDDK

COOPERATING UNITS (if any)

WRAIR (J.B. Long); MN-NINDS (M.A. Rogawski); Searle-Monsanto (P.C. Contreras); ARC-NIDA (R.B. Rothman); Istituto Superiore de Sanita (M. Iorio); Naval Research Lab (C. George); U of Illinois (M. Reith); Neurogen (A. Thurkauf).

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

Drug Design and Synthesis Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 7.0

PROFESSIONAL: 6.0

OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Among the CNS-active compounds which we have studied are ligands which interact with the PCP (phencyclidine) site, and those which interact with the sigma receptor. Our new, very high affinity and site selective sigma ligands will help elucidate the function of that receptor system.

Ligands for the PCP site: Studies in progress towards the design and synthesis of new PCP-like compounds as neuroprotective agents and as anticonvulsants. PCP-like compounds have been reported to exert a protective effect against neuronal degeneration in ischemia models; evidence suggests they act as antagonists against the depolarizing action of N-methyl-D-aspartate (NMDA) in animal brain. PCP binding sites exist in excitatory amino acid controlled ion channels regulated by glutamate receptors of the NMDA type, as well as in the dopamine uptake complex. Our initial electrophilic affinity ligand, metaphit, was an essential tool for the determination of dopaminergic neurotransmission in rat striatal slices and the involvement of the dopamine transporter and voltage-dependent sodium channel. Computer-assisted molecular modeling (CAMP) was used to examine the relative activities of isomeric methyl-substituted PCP isomers, one of which we determined to be among the most potent known PCP-like compounds. The CAMP study was based on a least squares fit to the pharmacophore and the calculation of the stability of the minimum energy conformer with the phenyl axial orientation. Future determination of the spatial area required by the macromolecule involved in binding should enable more accurate prediction of the activity of ligands.

Ligands for the sigma receptor: Sigma receptors are non-dopaminergic, non-opioid receptors which bind antipsychotic drugs and have been implicated in neural regulation of motor behavior and modulation of transmitter release upon electrical stimulation of smooth muscle preparations. We have identified a novel class of high-affinity sigma receptor ligands, the enantiomeric N-substituted cis-N-[2-(3,4-dichlorophenyl) ethyl]-2-(1-pyrrolidinyl)cyclohexylamines. The most potent, site selective, sigma ligand found in this group was the 1R,2S-(-)-unsubstituted derivative ($K_i=0.49$ nM). Related compounds constitute a new class of superpotent sigma ligands. Optically pure [3H](+)-cis-N-(2-(4-azidophenyl) ethyl)-2'-hydroxy-2,6-dimethyl-6,7-benzomorphan was synthesized and characterized as a high affinity and highly selective benzomorphan-based photoaffinity label for sigma receptors.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 59601-05 LMC

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analogues of Nucleic Acids and Their Components as Potential Anti-AIDS Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. F. Torrence

Section Chief

LMC/NIDDK

Others:

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

Biomedical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐

(a) Human subjects

☐

(b) Human tissues

☐

(c) Neither

☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Project terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 59602-18 LMC

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interferon Induction and Action. The Antiviral Action of Nucleoside Analogues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. F. Torrence Section Chief LMC/NIDDK

Others: T. Kovacs Visiting Associate LMC/NIDDK
W. Folkman Visiting Associate LMC/NIDDK
S. Khamnei Visiting Fellow LMC/NIDDK

COOPERATING UNITS (if any)

R. Silverman, Cancer Biology, Cleveland Clinic Foundation, Cleveland, Ohio

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

Biomedical Chemistry

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 4.0

PROFESSIONAL: 4.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Interferon induced enzyme activities such as the 2-5A synthetase, the 2',5'-phosphodiesterase, and the 2-5A-dependent ribonuclease are studied with the goal of understanding their role in the action of interferon, the induction of interferon by ds-RNA, and the control of cell growth and differentiation. Analogues of the mediator of interferon action are synthesized in order to define the relationship between oligonucleotide structure and binding to and activation of the 2-5A-dependent endonuclease. The eventual goal is to understand the biological role of the 2-5A system and to explore the potential of exploitation of this system in chemotherapy. Finally, a number of new approaches to pharmacologically active nucleoside analogues are pursued.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 59-701 19LMC

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Reactions and Immunochemistry of Carbohydrates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Cornelis P. J. Glaudemans Section Chief, SOC LMC/NIDDK

Others:	R. S. Arrepalli, E. M. Nashed	Visiting Scientist	LMC/NIDDK
	P. Kovac, E. Petrakova	Research Chem., Visiting Associate	LMC/NIDDK
	L. Mulard	Visiting Fellow	LMC/NIDDK
	V. Pozsgay	Visiting Scientist	LDMI, NICHD

COOPERATING UNITS (if any)

J. B. Robbins, NICHD	V. Kovacic, Czechoslovakia
R. Schneerson, NICHD	E. A. Kabat, Columbia University

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

Section on Carbohydrates

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 7.0

PROFESSIONAL: 7.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Section works on the interaction of (complex) carbohydrate determinants with monoclonal antibodies (MAbs). The elucidation of this interaction - in great molecular detail - is important since it pertains to all ligand-protein interactions. Thus, drug-receptor, effector-receptor as well as viral-receptor interactions may be clarified. We are executing:

1. Physico-chemical studies on antibody/antigen systems.
2. The synthesis of ligands for affinity studies.
3. The manipulation of immunoglobulin genes to produce specifically mutated genes expressing altered antibodies.
4. The study of immunodeterminants of bacteria causing significant diseases on a global scale, so as to evaluate procedures for vaccine development.

We have determined the specific interaction between microbial polysaccharides such as dextran and a number of monoclonal antibodies in the past. We have prepared many complex fragments of the capsular polysaccharide of *Shigella dysenteriae* type 1 by sophisticated syntheses, and have mapped the binding area of a monoclonal antibody towards this disease-causing micro-organism. In this manner, the immuno-determinant of this polysaccharide could be defined.

Both the variable region of the heavy (VH) and the light (VL) chains have been cloned and sequenced, and they have been incorporated in a bacterial expression vector. The VH and VL are linked by a short DNA sequence coding for a fifteen amino acid peptide so that the expressed protein is a covalently linked FV. Expression is presently going on in *E. Coli*.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 59801-01 LMC

PERIOD COVERED

October 1, 1991 through September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evaluation of Potential Cocaine Antagonists

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J.R. Glowa

Expert, Behavioral Pharmacol. Unit

LMC/NIDDK

Others:

COOPERATING UNITS (if any)

NIMH (E.M. Sternberg, M.A. Smith); NIAMS (R. Wilder); American University (A. Riley); NIDA (R. Rothman); CC-NM (P. Herscovitch).

LAB/BRANCH

Laboratory of Medicinal Chemistry

SECTION

Biomedical Chemistry

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 1.0

PROFESSIONAL: 1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Behavioral Pharmacology Unit is directed toward the assessment of pharmacological agents designed to modify the behavioral effects of substance abuse, using monkey self-administration and drug discrimination. Other capabilities have been developed to explore pharmacological properties of CNS-active drugs, in an effort to have access to rodent models to screen effects of agent before testing in primates. The neuroendocrine and immune function in plasma of drug-trained monkeys will be examined in the future, and PET scanning will be utilized before and after exposure to drugs of abuse in order to determine potential changes in various neurotransmitter systems as a consequence of prior drug exposure. These studies are unique in that they will investigate potential differences in animals working for drug, as opposed to simple non-contingent exposure to drug. Previous studies have linked susceptibility to drug abuse with individual differences in susceptibility to stress activation of the hypothalamic pituitary adrenal (HPA) axis. The primary endogenous agents involves in the HPA axis are corticotropin releasing hormone (CRH), adrenocorticotrophic hormone and cortisol. Several studies have been directed at examining the behavioral specificity of the direct effects of CRH on behavior.

Behavioral effects of beta-carbolines: These drugs exhibit profound behavioral effects, and may possibly serve as a pharmacological model of stress or anxiety. Our results with these drugs suggest that some behavioral effects of inverse agonists may not be strictly opposite those of benzodiazepines.

Effects of sigma ligands: Excitatory amino acids, acting at the NMDA receptor, have been postulated to play an important role in the acquisition of behavior (learning). Our data are consistent with the notion that excitatory amino acid blockade prevented the development of a learned emotional response, suggesting a potential role for this receptor in the development of anxiety-related disorders in humans.

Behavioral consequences of HIV infection - the role of GP120 in dementia: The effects of recombinant and purified native gp 120, the envelope protein of human immunodeficiency virus (HIV), were assessed on the acquisition of a spatial discrimination. It was found that gp120 may induce cognitive impairment in HIV-infected individuals, possibly through the interference of vasoactive intestinal polypeptide-mediated activity in the central nervous system.

PHOENIX EPIDEMIOLOGY AND CLINICAL RESEARCH BRANCH

Introduction

The Branch conducts epidemiologic and clinical research relating to the origin, development and natural history of non-insulin-dependent diabetes and its complications, and obesity, particularly among the Pima Indian population of Arizona. The Branch is based in Phoenix, Arizona and the facilities include a field clinic located in the Hu Hu Kam Memorial Hospital at Sacaton, Arizona on the Gila River Indian Reservation and the clinical research center in the Phoenix Indian Medical Center in Phoenix, Arizona. The Branch consists of three sections, the Clinical Diabetes and Nutrition Section, which conducts clinical research and laboratory investigations, the Diabetes and Arthritis Epidemiology Section, which is involved primarily with the epidemiological and genetic studies in the Pima Indian population residing on the Gila River Indian Reservation and the Biometry and Data Management Section which provides data management and support services for the Branch. The Branch also serves as a WHO Collaborating Center in Diabetes assisting other units and collaborating centers in methodology, design and analysis of epidemiological and clinical research studies in non-insulin-dependent diabetes mellitus.

The longitudinal study of the development of diabetes conducted by the Clinical Diabetes and Nutrition Section (CDNS) since 1982 has previously shown that insulin resistance is the first detectable abnormality that ultimately leads to non-insulin-dependent diabetes mellitus (NIDDM). Some 40 subjects have now developed diabetes out of the approximately 300 subjects who entered this study. The increasing numbers of subjects developing diabetes have now made enabled us to demonstrate among the subgroup of the population with marked insulin resistance that a lower acute insulin response is a further predictor of the likelihood of decompensation as previously predicted. This lends further support to the hypothesis that the development of non-insulin-dependent diabetes occurs in two stages, first the development of insulin resistance and, secondly, the development of an inadequate compensatory response by the beta cells of the pancreas to the continuing of and often increasing level of insulin resistance.

The molecular and biochemical basis of insulin resistance continue to be explored. Based on previous findings of reduced insulin-stimulated glycogen synthase activity in skeletal muscle of insulin resistant subjects, studies of the enzymes responsible for activating glycogen synthase have been pursued. A reduced level of basal activity of protein phosphatase, an enzyme responsible for activation of glycogen synthase has been found in insulin resistant subjects and additional isoforms of this enzyme in skeletal muscle have been recognized by the Section. Insulin resistance, however, is also accompanied by abnormalities in insulin activation of S6 kinase and abnormalities in the insulin regulation of MRNAs of several other proteins. Because a number of these pathways are abnormal in insulin resistant subjects the Section has recently focused on the possibility of abnormalities in the protein tyrosine phosphatases, enzymes which inhibit the activity of insulin receptor tyrosine kinase on various substrates. Insulin resistant subjects have been shown to have increased basal protein tyrosine phosphatase activity which fails to suppress in response to insulin infusion. There are, however, several protein tyrosine phosphatases present in skeletal muscle, and work to determine which of these isoforms is abnormal in insulin resistant subjects is underway.

Previously reported evidence of genetic linkage between insulin resistance and a locus on chromosome 4q has been extended. Evidence for linkage with all known polymorphic probes on the long arm of chromosome 4 has been sought and the locus for the site which is related to insulin resistance has been narrowed to within μ 5 centimorgans distance in the region 4q26 and probably lies between the locus for ANX5 and FABP2. In order to attempt to identify the specific gene involved, several overlapping YAC clones of this region are presently being examined to identify polymorphic sequences in this region and ultimately the specific gene in this region which is responsible for the observed genetic linkage with insulin resistance.

Obesity is an important determinant of the risk of developing non-insulin-dependent diabetes. Among the Pima Indians familial aggregation of several traits associated with the development of obesity have been identified, that are associated with abnormalities of metabolic rate. Recent studies have shown that reduced body temperature and reduced sympathetic nervous system activity are associated with obesity and reduced metabolic rate in the Pima. Direct measurement of sympathetic outflow has shown reduced activity among the Pima as compared to Caucasians, and we hypothesize that this abnormality and its associated reduction in metabolic rate may predispose to the development of obesity among the Pima.

Studies of muscle lipoprotein lipase activity, which is inversely related to the respiratory quotient have also been conducted to explore the possibility that abnormalities in lipid oxidation may be present in those who will subsequently become obese. These investigations are based on the fact that while increased carbohydrate and protein intake lead to increases in oxidation of these substrates, in contrast, an increased intake of fat is not accompanied by an increase in fat oxidation. The lack of such a response would lead to obesity if fat intake were excessive.

The longitudinal studies of risk factors for diabetes and vascular complications in the total Pima Indian community residing on the Gila River Indian Reservation conducted by the Diabetes and Arthritis Epidemiology Section has been continued. During the past year particular emphasis has been placed on testing and assembling families that are potentially informative with respect to the genetics of NIDDM itself, and also to diabetic nephropathy, which the section has previously shown to aggregate within diabetic families. Lymphoblast cultures have been established in a high proportion of living relatives within such families. DNA from such families is now being extensively probed for genes that relate to diabetes itself, insulin resistance, and diabetic renal disease.

These activities are described more fully in the individual section summaries.

Awards, Invited Lectures and Other Activities

P.H. Bennett, M.B., F.R.C.P., F.F.C.M.

European Association for the Study of Diabetes Claude Bernard Lectureship for Distinguish Contributions to Diabetes Research.

Editorial Activities

Consultant Editor for Diabetes, and for Diabetes and Metabolism Reviews; Advisory Board Acta Diabetologica; Associate Editor Journal of Public Health Medicine; and American Journal of Epidemiology.

Foreign Activities

Invited Speaker, South Pacific Commission, Diabetes Course, Noumea, New Caledonia, October 21-25, 1991

Guest Speaker, Israel Diabetes Association, Tel-Aviv, Israel, January 27-29, 1992

Invited Speaker, Society for Endocrinology, Metabolism, and Diabetes of Southern Africa, Durban, South Africa, March 3-6, 1992

Invited Speaker, University of CapeTown, CapeTown, South Africa, March 9, 1992

Invited Speaker, University of Bloemfontein, Bloemfontein, South Africa, March 10, 1992

Invited Speaker, University of Withwaterstrand, Johannesburg, South Africa, March 12, 1992

Invited Speaker, Sigrid Juselius Foundation Symposium on Genetic Epidemiology of Cardiovascular Disease in Diabetes, Helsinki, Finland, June 1-4, 1992

Other Activities

Buerki Visiting Professor, Henry Ford Hospital, Detroit, Michigan, November 6-7, 1991

Kelly West Memorial Lecture, University of Oklahoma, Oklahoma City, Oklahoma, April 7, 1992

Invited Speaker, Christopher Columbus Quincentenary Meeting: Aging-The Quality of Life, Washington, D.C., February 10-11, 1992

Clinical Diabetes and Nutrition Section

The scientific mission of the section is to determine the etiology and pathogenesis of non-insulin dependent diabetes mellitus (NIDDM) as it occurs among the Pima Indians of Arizona. To achieve this goal our major effort has been a cross-sectional and longitudinal study of non-diabetic Pima Indians to determine the metabolic factors that are predictive of the subsequent development of diabetes and how those factors change during the progression from normal glucose tolerance to NIDDM. To date, these studies have indicated that insulin resistance is the major risk factor for the development of NIDDM in the population and that a relatively low insulin secretory response is an additional minor risk factor. In addition, obesity is an independent risk factor for the development of the disease, which possibly acts by worsening insulin resistance. As a result of these observations, we are now attempting to identify the mechanisms of insulin resistance in the population at the biochemical and molecular level. In addition, we are studying factors that may contribute to individual variations in the insulin secretory response. Finally, since obesity is also a major risk factor for the disease, we are studying causes of obesity in the population.

Cross-sectional and longitudinal study of the development of NIDDM

A cross-sectional and longitudinal study of a subset of the Pima Indian population was initiated in 1982 with the specific aims of: 1) to identify the metabolic characteristics which are predictive of the development of NIDDM, and 2) to document the sequence of metabolic events that occur during the transition from normal to impaired glucose tolerance to NIDDM. Approximately 300 subjects have been entered into the study and are restudied annually. Today, approximately 40 individuals have developed NIDDM during the course of these studies.

Risk factor analyses of these data indicate that obesity is an important risk factor for the development of the disease, as previously observed in the population-based study. In addition, the analyses demonstrate that insulin resistance is a major risk factor for the development of NIDDM and that this effect is additional to the effects of obesity. Furthermore, among insulin resistant subjects, those with a lower acute insulin response have a slightly greater risk of developing the disease. These data suggest that the reason there is such a high prevalence of NIDDM in the Pima population is their greater degree of insulin resistance. The insulin resistance is a result not only of the greater prevalence of obesity in the population, but results from other factors, such as possible genetic determinants.

Mechanisms of insulin resistance. Since insulin resistance is a major risk factor for the development of NIDDM in the population, we have pursued studies of the underlying mechanism of insulin resistance using physiologic experiments, combinations of in vivo and in vitro studies, and genetic approaches.

Since recent studies in experimental animals suggest there is a large arterial/interstitial fluid insulin gradient, we undertook studies to determine whether this gradient exists in humans and whether it is greater in subjects with insulin resistance. Techniques were developed to simultaneously measure arterial and interstitial fluid insulin concentrations during insulin infusion. The results of these studies indicate that there is a large arterial/interstitial fluid insulin gradient in humans, but that the magnitude of this gradient is similar in insulin resistant and insulin sensitive subjects.

Previous in vivo experiments have demonstrated that a major tissue of insulin resistance in the Pima population is at the level of skeletal muscle. Studies have therefore been undertaken to study the insulin regulation of skeletal muscle metabolism by performing insulin infusions with simultaneous percutaneous muscle biopsies of the vastus lateralis to obtain tissue for in vitro analysis. In the past, these studies have demonstrated that there are several characteristics of muscle from insulin resistant subjects that are normal under basal conditions and in response to insulin. These included insulin binding, insulin receptor tyrosine kinase activity and casein kinase II activity. Abnormal responses have also been found; in particular, the insulin stimulation of glycogen synthase activity is reduced in insulin resistant subjects. This has been further explored by studying the activity of protein phosphatase I (PP1) which is the enzyme that dephosphorylates and activates glycogen synthase. The basal activity of this enzyme is reduced in insulin resistant subjects as is the response to insulin. Ongoing studies of this enzyme indicate that, in contrast to previous dogma, there is more than just the PP1 alpha form of the enzyme in human skeletal muscle. Which of these isoforms is most abnormal in insulin resistant subjects is under study.

In addition to the abnormalities of glycogen synthase activation and PP1 activity, abnormalities of insulin activation of S6 kinase and abnormal insulin regulation of mRNAs of several proteins have been found. Thus, there appear to be many abnormally regulated metabolic pathways in skeletal muscle of insulin resistant subjects. We therefore have focused attention on the protein tyrosine phosphatases (PTPases) which may inhibit the action of the insulin receptor tyrosine kinase on several substrates. The basal PTPase activity of insulin resistant subjects (in the particulate fraction of muscle biopsies) is increased, and, following insulin infusion, there is a suppression of soluble PTPase activity in normal subjects that is not observed in insulin resistant subjects. This suggests that abnormalities of PTPase activity may inhibit the tyrosine kinase activity of the insulin receptor and inhibit the insulin signal transduction in insulin resistant subjects. We have identified several PTPases in human skeletal muscle and which of these is the insulin regulated form is now under investigation.

In addition to these classical, biochemical approaches to understanding insulin resistance, we have also begun a molecular genetic approach to identify potential genetic abnormalities leading to insulin resistance in the Pima Indians. Initial studies indicated possible linkage between the glycophorin A/B locus on chromosome 4q (determining the MNSS blood groups) and insulin action in the Pimas. This was confirmed by detecting linkage between other markers of chromosome 4q, in particular FABP2 and ANK5 in the region of 4q26. There is no known candidate gene for insulin resistance in this region so we are using yeast artificial chromosomes to obtain DNA from this region to identify a possible candidate gene for insulin resistance. In addition, we are searching for other genetic determinants of insulin resistance by looking for linkage with various markers on chromosome 19 and chromosome 11.

Mechanisms of reduced insulin secretory responses. Since individuals with a relatively low acute insulin response in this population have a slightly increased risk of developing NIDDM, we have also been interested in identifying factors that determine individual variation in insulin secretory responses. The phenotype we have used is the acute insulin response to an acute intravenous

bolus of glucose. Acute insulin response aggregates families and has a frequency distribution that is quite different from a unimodal distribution. These data suggested that there may be genetic determinants of the acute insulin response.

To search for possible genes determining the acute insulin response, we have performed sibpair linkage analyses with various candidate genes. The initial studies indicate no linkage between the glucokinase locus and the acute insulin response. There are, however, some preliminary indications of possible linkage between the GLUT2 locus (glucose transporter) and the acute insulin response in the Pimas. Studies are now underway to screen a large number of individuals with a high or low acute insulin response for mutations of a GLUT2 exon by using the single stranded confirmation polymorphism (SSCP) technique.

Obesity. Obesity is extremely prevalent among the Pima Indians and is an important risk factor for the development of NIDDM, mostly due to its worsening effect on insulin sensitivity. For that reason, a major focus has been to identify possible metabolic factors leading to the development of obesity in the Pima population. Since we found that metabolic rate is a familial trait and that a low relative metabolic rate is a risk factor for weight gain, we have sought to identify the major determinants of metabolic rate. Using the indirect calorimetry method, both in a respiratory chamber and in ventilated hood systems, we found that the metabolic rate varies between individuals more than can be explained by individual differences in body size, body composition, age and sex. Interestingly, females have lower metabolic rates than males independently of differences in body size and body composition. Independently of the differences in body weight, body composition, age and sex, we also found that both body temperature and sympathetic nervous system (SNS) activity are related to metabolic rate. Using microneurone recording, we recently identified that Pima Indians have lower sympathetic nervous outflow compared to Caucasian volunteers. In both races, in response to body weight gain, there is an increase in SNS activity which is much smaller in Pima Indians than in Caucasians. Finally, this new technique has confirmed previously collected data showing that SNS activity is related to metabolic rate in Caucasians, but not in Pima Indians. This new line of investigation has yielded the first major difference which we have found in possible etiological factors for obesity in Pima Indians compared to Caucasians.

Growing evidence indicates that carbohydrate and protein stores are closely controlled since increasing the intake and of these nutrients stimulates their oxidation rates proportionally. Thus, chronic imbalance between intake and oxidation of non-fat nutrients cannot lead to obesity. On the other hand, fat stores are not controlled and the capacity for expansion is enormous. Since an increase in fat intake does not stimulate fat oxidation during spontaneous overfeeding, one can state that obesity is due mostly to long standing positive fat balance which may be due either high fat intake or impaired fat oxidation. In longitudinal studies, we have found that a low ratio of fat to carbohydrate oxidation is a risk factor for subsequent body weight gain. We therefore examined possible mechanisms involved in the storage and the oxidation of lipids in the body. We found that skeletal muscle lipoprotein lipase (LPL) activity correlated inversely with 24-hour respiratory quotient indicating that those subjects with a low fat to carbohydrate oxidation ratio had also a low skeletal muscle LPL activity. Muscle LPL activity may be a regulating factor of the skeletal muscle fatty acid uptake and oxidation and therefore might play a role in the etiology of obesity. Our findings also suggest that differences in muscle fiber types can account for part of the interindividual variability in muscle oxygen uptake and that muscle mitochondrial oxidative and high energy phosphate metabolism enzymes are inversely related to the ratio of carbohydrate to fat oxidation. The role of SNS activity on fuel utilization remains to be established.

In many experimental models, a low metabolic rate relative to body size is associated with hyperphagia. For that reason, we have initiated studies of ad libitum intake using an automated food selection system (vending machines). When subjects are exposed to a wide choice of food items they tend to spontaneously

overeating causing a positive fat balance and increased fat stores. The number of subjects studied is presently not sufficient to assess the relationship between energy expenditure and food intake. We plan to conduct a study of food intake and its relationship to fuel utilization comparing a low vs. a high fat diet using these vending machines. Finally, due to the strong genetic basis of obesity and to an association between blood groups (ABO) and body mass index in the Pima population, we plan to initiate a molecular genetic approach to identify genetic abnormalities leading to obesity in the population.

Invited Lectures and Invited Participation in Symposia

Clifton Bogardus, M.D.

"Impaired Skeletal Muscle Glycolysis as a Cause of Insulin Resistance in Man", Mid-Atlantic Research Symposium, Bethesda, Maryland, September, 1991

"Etiology and Pathogenesis of NIDDM", East Orange VA Medical Center, East Orange, New Jersey, January, 1992

"Causes of Insulin Resistance in the Pathogenesis of NIDDM", East Orange VA Medical Center, East Orange, New Jersey, January, 1992

"Genetics of Non-Insulin Dependent Diabetes and Obesity", Western Metabolism Club Conference, Carmel, California, February, 1992

"Obesity, Genetics and Insulin Resistance as Risk Factors for Type II Diabetes", AGING: The Quality of Life Conference, Washington, D.C., February, 1992

Eric Ravussin, Ph.D.

"Energy Metabolism in Obesity" and "Energy Balance vs. Nutrient Balance at Carbohydrate in our Lives", South African Sugar Association, Drakensberg, South Africa, September, 1991

"Energy Metabolism and Fuel Utilization in Obesity", Sixth Asian Congress of Nutrition, Kuala Lumpur, Malaysia, September, 1991

"Energy Expenditure and Pathophysiology of Obesity", Sixth Asian Congress of Nutrition, Kuala Lumpur, Malaysia, September, 1991

"Genetic Factors in Obesity and Insulin Resistance: Lessons learned from the Pima Indians", VIII Brazilian Diabetes Congress, Fortaleza, Brazil, 1991

"Obesity and Thermogenesis: Update", IV International Symposium of Obesity, Salvador, Bahia, Brazil, November, 1991

"Energy Metabolism in Obesity", American College of Nutrition, Clearwater, Florida, October, 1991

"Fuel Utilization in Obesity", First Maastricht Nutrition Symposium, Maastricht, The Netherlands, February, 1992

"Determinants of Energy Expenditure in Man: Emphasis on Sympathetic Nervous System Activity", University of Auckland, Auckland, New Zealand, July, 1992

"Energy Metabolism in Obesity: Calorie vs. Nutrient Balance", Australasian Society for the Study of Obesity Inaugural Scientific Meeting, Sydney, Australia, July, 1992

"Obesity and Diabetes: Lessons from the Pima Indians", Australasian Society for the Study of Obesity Inaugural Scientific Meeting, Sydney, Australia, July, 1992

Diabetes and Arthritis Epidemiology Section

The Diabetes and Arthritis Epidemiology Section has continued the longitudinal studies of genetic and environmental risk factors for diabetes and its vascular complications in the Pima Indians, as well as continuing epidemiologic studies of arthritis, cholelithiasis, mortality rates, and causes of death.

Genetics of NIDDM. The long follow-up provided by this study yields increasingly valuable data on complications of diabetes and the transmission of susceptibility to diabetes and its complications from one generation to the next. At least part of the susceptibility to diabetes appears to be transmitted by a major autosomal gene, but there is also evidence for effects of other genes and for nongenetic factors such as obesity and physical inactivity. To identify susceptibility genes, in collaboration with the CDNS, DNA samples have been collected and EBV-transformed lymphocyte cultures have been established from members of families informative for linkage analysis. Linkage analysis of DNA polymorphisms indicates the existence of a gene on chromosome 4q influencing insulin resistance and susceptibility to diabetes.

Glucose Intolerance in Pregnancy. The study of glucose tolerance in pregnant women, combined with regular follow-up of these women and their offspring, yields data on the long-term effects of diabetes and impaired glucose tolerance in pregnancy. Offspring of diabetic women had higher rates of obesity at every age than offspring of nondiabetic and prediabetic women. This finding was more marked in the younger age groups, suggesting that the effects of the diabetic pregnancy are seen mainly in childhood and adolescence and that other causes of obesity become more important in early adulthood. There is little difference in rates of obesity between offspring of nondiabetic and prediabetic women at any age. Few cases of diabetes developed before the age of 10 years. However, in older age groups, the offspring of diabetic women had a much higher prevalence of diabetes than either of the other two groups. Before the age of 25 years, the offspring of nondiabetic and prediabetic women had similar rates of diabetes. Thus, the metabolic abnormalities associated with the diabetic pregnancy result in long-term effects on the offspring including obesity and diabetes which in turn may contribute to transmission of risk for developing the same problems to the next generation.

Pima Indian women receiving an oral glucose tolerance test during pregnancy were tested again an average of 10 years later. Non-insulin-dependent diabetes developed three times as frequently in women who had had a 2-hour post-load plasma glucose concentration of at least 140 mg/dl than in women with lower post-load glucose concentrations. However, women who developed impaired glucose tolerance during pregnancy were at a lower risk of developing diabetes than were women who had impaired glucose tolerance when not pregnant.

Vascular Complications. Major diabetes complications under study are nephropathy, end stage renal disease, retinopathy, coronary heart disease (CHD), and lower extremity amputation. All of these complications except CHD develop at least as frequently in the Pima Indian population with NIDDM as in people with insulin-dependent diabetes. Glycosylated hemoglobin was found to be very effective in screening for diabetes (as defined by World Health Organization criteria) and in predicting complications in persons with diabetes. Because of its ease of sampling in outpatient conditions (requiring only a single, non-fasting blood sample) it can be more widely used for diabetes detection.

Patients with more extreme hyperglycemia at the diagnosis of diabetes are more likely to require insulin treatment during the course of their disease, which in turn indicates a higher risk of most vascular complications. This suggests that diabetes in this population may be heterogeneous in terms of its severity and risk of complications. The influence of the severity of hyperglycemia at diagnosis and of blood pressure prior to diagnosis of diabetes

on subsequent risk of nephropathy suggest that the course of diabetes is determined in part by conditions existing at or even before the onset of diabetes.

In collaboration with investigators from The Cleveland Clinic and Stanford University, we are performing a detailed study of renal function in Pimas with normal or impaired glucose tolerance or diabetes of short or long duration. These groups are compared with respect to glomerular filtration and pore size distribution, and these functional measures will be related to blood pressure, degree of hyperglycemia, and disease progression during subsequent follow-up. The study indicates a defect in glomerular pore size is presently early in the course of diabetes without a major change in glomerular filtration rate.

Other Activities. Section staff continue to be active in medical research and education beyond the projects described here. Staff collaborate extensively in research projects conducted by the CDNS and the National Center for Health Statistics. They continue lecturing at universities and contribute to national and international meetings and workshops.

Invited Lectures and Invited Participation in Symposia

William C. Knowler

Visiting Professor, Department of Community Health Sciences, Lund University, Dalby, Sweden (supported by the Swedish Medical Research Council), 1990-

Pathophysiology and Genetics of NIDDM and its Complications in the Pima Indians, Workshop on Diabetes in Minority Populations, University of Hawaii at Manoa, Honolulu, Hawaii, November 14-16, 1991

The Genetics of Type II Diabetes, 4th International Diabetes Conference: Current Topics in Diabetes Research, Florence, Italy, March 18, 1992

David J. Pettitt

Diabetes and pregnancy - What we have learned from the Pima studies. Diabetes in Alaska 1991. Alaska Native Medical Center, Anchorage, Alaska, December 4, 1991

Landmark studies of diabetes in Native People - The Pima studies. Diabetes in Alaska 1991. Alaska Native Medical Center, Anchorage, Alaska, December 5, 1991

Report of Gestational Diabetes Research on Pimas - Genetic Predisposition and Intrauterine Environment as Risk Factors for Diabetes. Indian Health Service and Tribal health care providers' workshop, Phoenix, Arizona, January 29, 1992

The long-term effects of diabetic pregnancy on the offspring -The Pima Indian Experience. First International Symposium on Diabetes and Pregnancy in the 90's, Tel Aviv, Israel, April 2, 1992

Long term outcome of offspring of gestational and type II diabetics. Phoenix Indian Medical Center Clinical Staff Conference, Phoenix, Arizona, April 29, 1992

Abnormal glucose tolerance in Pima Indian Pregnancies: Implications for the mother and child. Diabetes Workshop at the 5th Annual IHS Research Conference, Tucson, Arizona, May 5, 1992

Abnormal glucose tolerance in Pima Indian Pregnancies: Implications for the mother and child. Workshop for the Oklahoma City Area Indian Health Service Diabetes Multidisciplinary Committee, Oklahoma City, Oklahoma, August 20, 1992

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 69000-27 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Diabetes Mellitus and Other Chronic Diseases in the Gila River Indian Community

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

W.C. Knowler	Chief	DAES, NIDDK
P.H. Bennett	Chief	PECRB, NIDDK
D.J. Pettitt	Assistant Chief	DAES, NIDDK
M.A. Charles	Visiting Associate	DAES, NIDDK
D.R. McCance	Visiting Associate	DAES, NIDDK
R. Hanson	Medical Staff Fellow	DAES, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; National Institute of Arthritis and Musculoskeletal and Skin Diseases; Ariz. State U.; Cleveland Clinic, Cleveland OH; University of Pittsburgh.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

3.8

PROFESSIONAL:

2.4

OTHER:

1.4

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to identify the determinants of non-insulin-dependent diabetes (NIDDM), various types of arthritis, and gallbladder disease, and elucidate the natural history of the diseases. Genetic and environmental risk factors for NIDDM have been studied in the Pima Indians. The residents of the study area, approximately 5000 people, have participated in a longitudinal population study since 1965, allowing observations of the natural history of diabetes mellitus. Risk factors for obesity, hypertension, and cholelithiasis are also studied, along with the relationships of these diseases to diabetes and their effects on mortality rates. The genetics of diabetes is studied by means of family studies and relationships of genetic markers to disease. The roles of obesity, serum insulin concentrations, impaired glucose tolerance, occupational and leisure-time physical activity and diabetes in relatives are assessed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69001-23 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Complications and Outcome of Diabetic and Prediabetic Pregnancies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	W.C. Knowler	Chief	DAFS, NIDDK
	M.A. Charles	Visiting Associate	DAES, NIDDK
	R.L. Hanson	Epidem Staff Fellow	DAES, NIDDK
	D.R. McCance	Visiting Associate	DAES, NIDDK

COOPERATING UNITS (If any)

Indian Health Service; Biostatistics and Data Management Section, PECRB;
Mayo Clinic, Rochester, Minnesota (B.A. Kottke)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

1.3

PROFESSIONAL:

0.8

OTHER:

0.5

CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purposes of the project are to determine the effects of abnormal glucose tolerance on outcome of the pregnancy, to determine long term prognosis for the women and their offspring, and to identify diabetes and impaired glucose tolerance during pregnancy in women in the Gila River Indian Community. Women with impaired glucose tolerance during pregnancy were at a higher risk of developing diabetes within 10 years than were women with normal glucose tolerance. However, they were at a lower risk than women who developed impaired glucose tolerance in the absence of pregnancy. Infants of diabetic mothers are more likely than infants of nondiabetic mothers to exhibit macrosomia, prematurity, perinatal mortality, and congenital malformations. Offspring of diabetic women are also at an increased risk of developing obesity and glucose intolerance during childhood and young adulthood. By means of a glucose tolerance test as well as chart review, the diabetes status of every woman is determined at two-yearly intervals and during the third trimester of each pregnancy. The characteristics of women who have diabetes or impaired glucose tolerance during the pregnancy are compared with those of women who are normal during the pregnancy and subsequently develop diabetes and with those of women who remain normal. These women and their offspring, after the age of 5 years, are followed at two yearly intervals and glucose tolerance tests are performed which include measurements of glucose and insulin. The offspring of diabetic women had higher rates of obesity at every age than did offspring of nondiabetic and prediabetic women. This finding was more marked in the younger age groups, suggesting that the effects of the diabetic pregnancy are seen mainly in childhood and adolescence and that other causes of obesity become more important in early adulthood. There was little difference in rates of obesity between offspring of nondiabetic and prediabetic women at any age. Few cases of diabetes developed before the age of 10 years, however, in older age groups, the offspring of diabetic women had a much higher prevalence of diabetes than either of the other two groups. Before the age of 25 years, the offspring of nondiabetic and prediabetic women had similar rates of diabetes. Thus, the metabolic abnormalities associated with the diabetic pregnancy result in long-term effects on the offspring including obesity and diabetes which in turn may contribute to transmission of risk for developing the same problems to the next generation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69006-22 PCCR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gila River Indian Community Autopsy and Mortality Study

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P.H. Bennett	Chief	PECRB, NIDDK
Others:	W.C. Knowler	Chief	DAES, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	M.L. Sievers	Guest Researcher	DAES, NIDDK
	Q.Z. Liu	Visiting Fellow	DAES, NIDDK
	L. Striker	Chief	RCBS, NIDDK

COOPERATING UNITS (if any)

Pathology Department, Phoenix Indian Medical Center, Indian Health Service, Phoenix, Arizona; Cleveland Clinic Foundation, Phoenix, Arizona;

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The causes of death and postmortem characteristics of Pima Indians of the Gila River Indian Community are investigated so that findings in subjects with and without diabetes mellitus can be correlated with studies in living subjects. Medical records are reviewed for the determination of cause of death and for the occurrence of certain serious diseases or complications of diabetes.

The purpose of the study is to relate the outcome and cause of death to events or risk factors measured in life among Pima Indian residents of the Gila River Indian Community, particularly in relation to diabetes, cardiovascular diseases and gallbladder disease. Death Certificates are obtained on all members of the Gila River Indian Community. In addition, post mortem examinations and all available medical records pertaining to the subjects are obtained to ascertain conditions present at the time of death and ascertain cause of death as precisely as possible. These records are reviewed in a standardized way for evidence of the complications of diabetes, vascular disease, neoplasms and other conditions. The records are used to determine the causes of death and incidence of complications associated with diabetes and other conditions identified initially during life by the longitudinal epidemiologic studies in the population.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69009-27 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Natural History of Arthritis and Rheumatism in the Gila River Indian Community

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P.H. Bennett	Chief	PECRB, NIDDK
Others:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	W.C. Knowler	Chief	DAES, NIDDK
	S.P. Heyse	Chief	OPECA, NIAMS
	L. Jacobsen	Visiting Associate	OPECA, NIAMS

COOPERATING UNITS (if any)

Indian Health Service, Arizona State University; NIAMS

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

0.5

PROFESSIONAL:

0.3

OTHER:

0.2

CHECK APPROPRIATE BOXES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The development and progression of osteoarthritis, rheumatoid arthritis and ankylosing spondylitis are being determined by means of clinical, radiographic and serological examinations carried out prospectively at two-yearly intervals among adults of the Gila River Indian Community (Pima Indians) in Arizona, in conjunction with epidemiological studies of diabetes in the same community. The purpose of this investigation is to ascertain the determinants of these diseases in the population, and to identify factors which predispose to or alter the natural history of progression of the disease. Host factors such as age, sex, and various gene markers including HLA and Gm, together with various potential environmental determinants, such as obesity and evidence of exposure to infectious agents, are being investigated prospectively to determine their relationship to the development of these diseases. Longitudinal data have now been collected for 25 years and represent a unique data set for epidemiological studies of arthritis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69015-10 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cross-Sectional and Longitudinal Study of "pre-diabetes" in the Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	R. Ferraro	Staff Fellow	CDNS, NIDDK
	M. Spraul	Visiting Associate	CDNS, NIDDK
	D. Mott	Supervisory Res. Chem.	CDNS, NIDDK

COOPERATING UNITS (If any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

2.1

PROFESSIONAL:

1.35

OTHER:

.75

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrevoked type. Do not exceed the space provided.)

The Pima Indians of Arizona have the highest reported prevalence and incidence of non-insulin dependent diabetes mellitus (NIDDM) of any population in the world. Since 1982, we have been studying a subset of this population to determine the metabolic characteristics that predispose individuals in the population to develop the disease. Approximately 300 subjects were entered into the study and are followed yearly. Subjects are admitted to the clinical research ward and undergo several studies including underwater weighing, oral glucose tolerance test, intravenous glucose tolerance test, standard mixed meal test, and a two-step hyperinsulinemic, euglycemic clamp to measure insulin action in vivo. This past decade approximately 40 subjects have developed NIDDM during the course of this study. Analyses of the data collected to date indicate that insulin resistance is a major risk factor for the development of NIDDM, independent of the risk associated with being obese. In addition, among individuals who are insulin resistant a lower acute insulin response is an additional risk factor. However, those individuals with a low acute insulin response have acute insulin responses that are the same or higher as a matched group of Caucasian individuals. These data suggest that insulin resistance is the major risk factor for the development of NIDDM in the Pima population and is probably the explanation for the greater prevalence of the disease in this racial group. Among insulin resistant persons, a low acute insulin response is an additional risk factor for the development of NIDDM. Both insulin resistance and a low acute insulin response appear to cluster in families and both have a frequency distribution that is not fit by a unimodal distribution. These data suggest there may be genetic factors that contribute to insulin resistance and lower acute insulin responses in this population.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69020-08 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Resistance and the Regulation of Muscle Glycogen Synthase Activity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	H. Mori	Visiting Fellow	CDNS, NIDDK
	S. Norman	Visiting Fellow	CDNS, NIDDK
	D. Mott	Supervisory Res. Chem.	CDNS, NIDDK

COOPERATING UNITS (If any)

Indian Health Service; Second Department of Medicine, University of Helsinki, Helsinki, Finland (H. Yki-Jarvinen)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

2.8

PROFESSIONAL:

1.8

OTHER:

1.0

CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are currently characterizing the abnormalities for regulation of human muscle glycogen synthesis in insulin-resistant subjects. In insulin-resistant subjects, fasting glycogen synthase phosphatase and phosphorylase phosphatase activities are reduced and fail to show the peak insulin stimulation observed for insulin-sensitive subjects at 10-20 minutes. Using specific inhibitors the abnormal enzyme activities were identified as a type-1 phosphatase (PP-1) in human muscle from insulin-resistant subjects. The abnormally low fasting PP-1 activity in insulin-resistant subjects persisted following trypsin treatment, suggesting that inhibitors 1 and 2 (characterized regulators of PP-1) are not important determinants of the abnormal phosphatase activity. Western blots indicate an increased concentration of catalytic subunit for PP-1 in the muscle from insulin-resistant subjects. These results suggest that the intrinsic activity or regulation of the catalytic subunit is abnormal in insulin-resistant subjects. All insulin resistant subjects showed Mn activation of PP-1 in the absence of azide. 6 of 10 insulin sensitive subjects, however, required azide in order to see Mn activation of PP-1. The azide appears to reverse the effects of an inhibitor of Mn activation which has been localized in the glycogen-microsomal subcellular fraction of Mn-resistant (insulin sensitive) subjects. These results suggest that an azide sensitive structure in the glycogen microsomal fraction of muscle is responsible for the abnormal PP-1 activity in insulin resistant subjects. A second isoform of PP-1 has been identified in human muscle. Characterization of this isoform may explain the activity differences observed for insulin resistant and sensitive subjects. Compared to insulin sensitive subjects, subjects with NIDDM have elevated fasting PP-1 activity which, following insulin infusion, not only fails to increase but shows a significant decrease. Four weeks of insulin therapy reduces the elevated fasting PP-1 activity in NIDDM subjects and prevents their insulin mediated decrease in enzyme activity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 69021-11 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Energy Expenditure in Pima Indians: Risk Factors for Body Weight Gain

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E. Ravussin	Visiting Scientist	CDNS, NIDDK
Others:	R. Ferraro	Staff Fellow	CDNS, NIDDK
	R. Rising	IRTA	CDNS, NIDDK
	M. Spraul	Visiting Associate	CDNS, NIDDK
	R. Norman	Staff Fellow	CDNS, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Dept. of Medicine, Northwestern Medical School, Chicago, IL, (J. Young); Rockefeller University, New York, NY (R. Leibel)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

2.95

PROFESSIONAL:

2.7

OTHER:

0.25

CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Obesity is very prevalent among the Pima Indians and represents a major risk factor for insulin resistance and the development of NIDDM. Obesity is a condition of excess body fat and seems to be genetically determined. Our search for the causes of obesity in the Pima population has identified four known familial metabolic parameters predicting body weight gain: a low metabolic rate, a high respiratory quotient (low fat/carbohydrate oxidation ratio), insulin sensitivity, and a low spontaneous physical activity. We are therefore investigating the causes of the variability of these metabolic parameters: 1) females have lower metabolic rate and lower fat oxidation than males, even after adjusting for differences in body size and composition; 2) age is not a major determinant of sedentary energy expenditure, but is associated with lowering of physical activity in free-living conditions; 3) metabolic rate seems to be regulated to maintain a genetically determined body temperature and Pima Indians have lower a sleeping body temperature than Caucasians; and 4) sympathetic nervous system (SNS) activity, measured by microneurve recording is lower in Pima Indians than in Caucasians. In response to spontaneous overfeeding, carbohydrate and protein stores are closely controlled whereas increased fat intake does not result in increased fat oxidation. This has led us to study possible mechanisms regulating fat oxidation: 1) skeletal muscle lipoprotein lipase (LPL) activity correlated inversely with 24-hour respiratory quotient, indicating that a low skeletal muscle LPL activity might cause decreased fatty acid uptake and oxidation; 2) differences in muscle fiber types account for part of the interindividual variability in energy metabolism. These new observations suggest that muscle might be the site of differences in fat utilization which might predispose to obesity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69024-06 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

WHO Collaborating Center for Epidemiological and Clinical Investigations

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P.H. Bennett	Chief	PECR, NIDDK
Others:	W.C. Knowler	Chief	DAES, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK

COOPERATING UNITS (if any)

World Health Organization, Non-Communicable Diseases Program, Geneva, Switzerland, (Foreign), Other World Health Organization Collaborating Centers for Diabetes (Foreign), China-Japanese Friendship Hospital, Beijing, China

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

0.3

PROFESSIONAL:

0.1

OTHER:

0.2

CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrevoked type. Do not exceed the space provided.)

The Phoenix Epidemiology and Clinical Research Branch was designated as the WHO Collaborating Center for Design, Methodology and Analysis of Epidemiological and Clinical Investigations in Diabetes in 1986. The purposes of the Center are to collaborate with the World Health Organization in the implementation of the WHO/IDF action program to provide advice, consultation and collaboration with other investigators in the design, methodology and analysis of epidemiology and clinical diabetes (NIDDM) and its complications. The center will assist in the development and application of standardized methods for epidemiological and clinical investigations relating to the etiology and pathogenesis of non-insulin-dependent diabetes (NIDDM) and its complications. The center will assist in the development and application of standardized methods for epidemiological and clinical investigations, and data analysis relating to diabetes and collaborate with those interested in applying such techniques elsewhere. The Center will advise and help in the design of new studies, including on site assistance when necessary. The Center serves to train investigators from many parts of the world in diabetes epidemiology and clinical research.

The Center participates in the WHO Multicenter Study of Vascular Disease in Diabetes, which is examining the mortality and incidence of vascular complications of diabetes among different ethnic groups in different countries.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69025-06 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Treatment of Impaired Glucose Tolerance in Malmöhus County Sweden

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DARS, NIDDK

COOPERATING UNITS (if any)

Lund University, Dalby, Sweden (Foreign)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The prognosis and effect of treatment of impaired glucose tolerance (IGT) are studied in residents of Malmöhus County, Sweden. Mortality rates are determined in over 2000 persons who had glucose tolerance tests in the 1960s, and results are related to glucose tolerance and other factors at baseline. Some of these subjects participated in a randomized treatment study of IGT. New studies in population screening and treatment of IGT are being planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69028-04 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetics of Non-Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W.C. Knowler	Chief	DAES, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	P.H. Bennett	Chief	PECRB, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK
	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	M. Prochazka	Senior Staff Fellow	CDNS, NIDDK
	M.A. Charles	Visiting Associate	DAES, NIDDK

COOPERATING UNITS (if any)

Bowman-Gray Medical School, Winston-Salem, NC; Howard Hughes Medical Institute, University of Chicago, Chicago IL; Arizona State University, Tempe, AZ.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

1.2

PROFESSIONAL:

0.3

OTHER:

0.9

CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrevoked type. Do not exceed the space provided.)

Non-insulin dependent diabetes mellitus is a common chronic disease that develops in most populations in late middle age. The Pima Indians of Arizona have the highest reported prevalence of this disease in the world and in contrast to many populations the disease often presents at an earlier age. As a result of long-term epidemiological studies in the total population, the familial nature of the disease has been well documented, and segregation analyses suggest the possibility of inheritance by a single additive major gene.

This project will search for genetic determinants of NIDDM using the techniques of genetic linkage analysis with restriction fragment length polymorphism (RFLP) and other genetic markers to identify the chromosomal location of inherited determinants of NIDDM in the Pima Indian population. A number of informative pedigrees have been identified and lymphoblast cell lines from informative members of these pedigrees established. DNA from these lymphoblasts is isolated and polymorphic probes applied to search for evidence of linkage of these markers and NIDDM. Probes with established chromosomal locations will be used to screen the genome to detect genetic linkage with NIDDM as described in Project# Z01 DK 69045-01 (Molecular genetic analysis of a chromosome 4 region harboring agent controlling insulin action).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69029-04 PCCR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Regulation of Skeletal Muscle Ribosomal Protein S6 Kinase by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Sommercorn Senior Staff Fellow CDNS, NIDDK
Others: C. Bogardus Chief CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

.27

PROFESSIONAL:

.27

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The effect of insulin on S6 kinase activity in skeletal muscle from insulin sensitive and insulin resistant Pima Indians was examined. The S6 kinase was assayed in extracts of skeletal biopsies obtained from the vastus lateralis at 0, 15, 30, 45, 60, and 90 minutes during a hyperinsulinemic, (~2000 $\mu\text{U/ml}$) euglycemic clamp. Prior to the infusion of insulin S6 kinase activity was similar in the groups. In insulin sensitive subjects, S6 kinase activity increased between 15 and 30 minutes of insulin infusion reaching a maximum at 45 minutes and then declined. S6 kinase activity also increased in resistant subjects, but the maximum occurred later, at 60 minutes, without the rapid increase between 15 and 30 minutes. The absence of an earlier insulin response of S6 kinase suggested that insulin resistant subjects may lack a particular earlier responding S6 kinase. Chromatography of extracts on FPLC MonoQ revealed two peaks of insulin-stimulated S6 kinase activity that eluded similarly in the two groups. Most of the increased activity occurred in peak 2 and immunoblot analysis revealed that the enzyme responsible for peak 1 activity is antigenically related to the 90 kilodalton S6 kinase and the peak 2 activity, is accounted for by an enzyme antigenically related to the 70 kilodalton S6 kinase. The results suggest that the mechanism for insulin resistance in Pima Indians occurs upstream from the S6 kinase in the pathway of insulin signal transduction. The project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69030-04 PCCR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Contribution of Protein Tyrosine Phosphatase to Insulin Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Sommercorn	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	M. Kunkel	IRTA	CDNS, NIDDK
	J. Rowles	IRTA	CDNS, NIDDK
	N. Tonks	Senior Staff Scientist	Cold Spring Harbor Labs., CSH, N.Y.

COOPERATING UNITS (If any)

Indian Health Service; Cold Spring Harbor Labs., CSH, NY (N. Tonks)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

2.27

PROFESSIONAL:

2.27

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We previously found that insulin rapidly suppresses the activity of a soluble PTPase in skeletal muscle of insulin-sensitive subjects, but not in subjects resistant to insulin and that activity of a particulate PTPase is chronically elevated in muscle of resistant subjects. Either of these abnormalities could be responsible for insulin resistance. We have confirmed the rapid suppression of PTPase activity by insulin in cultured cells and in rabbit skeletal muscle in vivo. The insulin suppressed PTPase can be resolved by ion-exchange chromatography. As part of our effort to understand the contribution of PTPases to insulin resistance, we identified PTPase cDNAs in human skeletal muscle using PCR. The 5 major PTPases amplified included PTP1B, TCPTP, the band 4.1 - related PTPases, PTPH1 and PTP-MEG, and a novel putative PTPase. We have isolated a full-length cDNA and expressed the catalytic domain of this novel PTPase, which we call PTP-PEST because its C-terminal segment is rich in Pro, Glu/Asp and Ser/Thr and contains potential PEST sequences, which are found in proteins with very short intracellular half lives. PTP1B and PTP-PEST RNA species increase 3-5 fold in response to insulin, suggesting that these PTPases may contribute to long-term effects of the hormone.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69031-04 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Phosphorylase Phosphatase by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Sommercorn	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	S. Norman	IRTA	CDNS, NIDDK
	B. Thompson	Senior Staff Fellow	CDNS, NIDDK
	D. Mott	Supervisory Res. Chem.	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

.67

PROFESSIONAL:

.67

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Impaired activation of glycogen synthase in insulin-resistant subjects is associated with both chronically lower activity of protein phosphatase-1 (Pp1) and a smaller magnitude of activation of Pp1 in response to insulin compared to that seen in insulin-sensitive subjects. Although Pp1 activity is low in muscle of resistant subjects, the concentration of catalytic subunit of Pp1 (Pp1-C) is higher than in sensitive subjects (B. Nyomba and D. Mott, CDNS, PECRB). The predicted amino acid sequence of the catalytic domain of Pp1 was examined in insulin sensitive and resistant subjects, using polymerase chain reaction (PCR), and found to be identical. Pp1-C RNA concentration and size in skeletal muscle (1.6 kb) were also the same in fasting subjects of different insulin sensitivities. Therefore, it is unlikely that a major mutation in Pp1 accounts for abnormal properties of Pp1 activity in insulin resistance. The concentration of RNA corresponding to Pp1-C in human skeletal muscle decreased by 50% after 30 min of a high dose infusion of insulin in vivo. The concentration returned to basal levels by 120 min. In contrast, Pp1-C RNA increases slightly in response to insulin in insulin-resistant subjects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69036-03 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Epidemiology of Complications of Non-Insulin-Dependent Diabetes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler	Chief	DAES, NIDDK
P.H. Bennett	Chief	PECRB, NIDDK
D.J. Pettitt	Assistant Chief	DAES, NIDDK
Q.Z. Liu	Visiting Fellow	DAES, NIDDK
M.A. Charles	Visiting Associate	DAES, NIDDK
D.R. McCance	Visiting Associate	DAES, NIDDK
R. Hanson	Epidemiology Staff Fellow	DAES, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Cleveland Clinic Foundation, Cleveland, OH.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

3.4

PROFESSIONAL:

2.2

OTHER:

1.2

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of the project is to determine the incidence rates, rates of progression, and risk factors for the chronic complications of NIDDM. The study is conducted in the Pima Indians of the Gila River Indian Community, who have participated in a longitudinal epidemiologic study since 1965 (see project Z01 DK 69000).

Risk factors for the major complications of diabetes, retinopathy, nephropathy, coronary artery disease, and peripheral vascular disease are determined by longitudinal followup of diabetic subjects. Methods of ascertainment of these complications include fundus photography, measurement of urine albumin and serum creatinine concentrations, electrocardiography, and documentation of lower extremity amputations.

These complications are responsible for the major morbidity and excessive mortality associated with diabetes in this population. The incidence rates of severe complications such as end stage renal disease, cataract requiring surgical treatment, and lower extremity amputations are as high among diabetic Pimas as reported anywhere else in the world. Diabetic nephropathy is the leading cause of deaths among diabetic Pimas. Late stages of nephropathy can be predicted by minor abnormalities of urinary albumin excretion early in the course of the disease and show strong familial aggregation suggesting a genetic susceptibility to nephropathy as well as to diabetes. Development of nephropathy is predicted by elevated blood pressure before the onset of diabetes, suggesting that predisposition to diabetic renal disease is determined, in part, even before the onset of diabetes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69037-03 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Kidney Function in Non-Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett

Chief

PECRB, NIDDK

Others: W.C. Knowler

Chief

DARS, NIDDK

COOPERATING UNITS (If any)

Cleveland Clinic Foundation; Stanford University; Emory University; Chronic Renal Diseases Program, NIDDK

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

0.3

PROFESSIONAL:

0.2

OTHER:

0.1

CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The functional characteristics of the renal glomerulus are being investigated in Pima Indians of the Gila River Indian Community to identify the underlying pathogenetic mechanisms involved in the initiation and progression of renal disease in non-insulin-dependent diabetes mellitus (NIDDM).

The Pima Indian population has a high incidence of NIDDM and diabetic nephropathy. Six groups of subjects are being studied: subjects with normal glucose tolerance, individuals with impaired glucose tolerance (IGT), those with newly diagnosed diabetes (<3 years duration NIDDM), and diabetic subjects (≥5 years duration NIDDM) with evidence of (a.) mildly abnormal albumin excretion (b.) severe abnormalities of albumin excretion (c.) normal albumin excretion. Measurements of renal and glomerular capillary wall function including glomerular filtration rate (GFR), renal plasma flow (RPF), dextran sieving coefficients, and albuminuria are being performed and correlated. Markers and/or predictors of progression as well as the mechanisms of initiation and progression of diabetic renal disease are being sought.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69038-03 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin and Hypertension in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler	Chief	DAES, NIDDK
Q.Z. Liu	Visiting Fellow	DAES, NIDDK
D.J. Pettitt	Assistant Chief	DAES, NIDDK
P.H. Bennett	Chief	PECRB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOXES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

It has been suggested that subjects with diabetes or impaired glucose tolerance (IGT) have a higher prevalence of hypertension than subjects with normal glucose tolerance (NGT). Both obesity and glucose intolerance are related to increased insulin resistance, hyperinsulinemia, and increased risk of hypertension. Moreover, patients with essential hypertension are insulin resistant and hyperinsulinemic. Thus, it has been postulated that insulin might be involved in the pathogenesis of hypertension. The incidence of hypertension by glucose tolerance was determined in 2784 Pima Indians without clinical proteinuria at baseline, and the associations between insulin treatment or endogenous insulin concentration and incidence of hypertension were examined among these subjects.

Subjects with diabetes or IGT had a higher incidence of hypertension than did subjects with NGT. Among all diabetic subjects, exogenous insulin (insulin treatment) predicted subsequent hypertension. Among nondiabetic subjects, but not diabetic subjects treated with insulin, endogenous fasting insulin concentration predicted subsequent hypertension. Thus, these findings support the hypothesis that insulin plays a role in developing hypertension in non-diabetic Pima Indians.

THIS PROJECT HAS BEEN TERMINATED.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69039-03 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dietary Survey of the Pima Indians of the Gila River Indian Community

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DABS, NIDDK
P.H. Bennett Chief PECRB, NIDDK
D.J. Pettitt Assistant Chief DABS, NIDDK

COOPERATING UNITS (if any)

Cleveland Clinic Foundation, Phoenix, Arizona.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

An age-sex-stratified sample of 600 residents of the Gila River Indian Community, ages 18-75 years, was recruited for a dietary survey. Dietary intake was estimated by the dietary history method to obtain quantitative food frequency information. Reported energy intake was higher in men and negatively related to age; thus, relationships with weight were analyzed by multiple regression controlling for sex and age. Energy intake in kilocalories (kcal) was positively associated with body weight or body mass index, adjusted for age and sex. Body weight was associated with absolute intake of the major dietary components, carbohydrate, fat, and protein, but not with any of these components expressed per 1000 kcal, suggesting that total energy intake, rather than proportions of specific components, was the variable having the strongest association with body weight. Alcohol consumption (in any amount) was higher in men and inversely associated with age and weight. Neither energy intake nor specific components were significantly associated with diabetes, after adjustment for age and sex, nor did diabetes affect the relationship between energy and weight, in a subset of 307 subjects (153 diabetics) examined for diabetes within one year of the diet history. The acceptance of obesity in this population may reduce the under-reporting of energy intake which has been postulated in other studies. Body weight in Pima Indians is weakly but positively associated with increased caloric intake.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69040-03 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sodium-Lithium Countertransport and Blood Pressure

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DAES, NIDDK

D.J. Pettitt Assistant Chief DAES, NIDDK

COOPERATING UNITS (if any)

University of Pittsburgh; University of Southern California

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Red blood cell sodium lithium countertransport is a genetic marker of hypertension in several ethnic groups. It reflects the sodium-hydrogen antiport activity in renal tubules. Several recent studies showed an association between sodium-lithium countertransport and predisposition to diabetic nephropathy. We are studying the relationships between sodium-lithium countertransport, nephropathy, and blood pressure in a sample of 200 Pima Indians.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69041-03 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Resistance in Obesity and the Association with Lymph Insulin Kinetics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. Lillioja Visiting Scientist CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

.15

PROFESSIONAL:

.15

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have previously shown that the density of capillary supply in skeletal muscle of man correlates with insulin resistance. We postulated that since the unfenestrated capillaries of muscle are relatively impermeable to insulin that the increased insulin resistance in those with low capillary density might be due to altered kinetics of insulin penetration to its sites of action in muscle. We have published a method of directly collecting lymph from a peripheral lymphatic vessel in sufficient amounts to measure insulin, and glucose during changes in arterial insulin. Insulin concentrations in limb lymph are much lower than in plasma and in contrast to plasma are highly correlated with glucose uptake in each individual. However, compared to obese insulin resistant subjects, the slope of the glucose uptake/insulin relationship is much steeper in lean subjects. Hence, interstitial insulin concentrations determine insulin action but individual variations in insulin resistance are determined more distally in the pathway leading to insulin mediated glucose uptake.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69043-03 PECR

PERIOD COVERED

October 1, 1992 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Gene Expression by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Sommercorn	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	B. Thompson	Senior Staff Fellow	CDNS, NIDDK

COOPERATING UNITS (If any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

.37

PROFESSIONAL:

.37

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to identify genes that normally respond to insulin *in vivo* and specifically, to explore the pathway of insulin regulated gene expression in human skeletal muscle in individuals with different insulin sensitivities, we have adapted the S1 nuclease protection assay for use with multiple probes (multiple S1 nuclease protection assay) to allow the simultaneous examination of RNA abundances from multiple genes. In conjunction with hyperinsulinemic, euglycemic clamp technique, we have evaluated the ability of insulin to alter the RNA abundances of several proto-oncogenes and on other potentially insulin responsive genes in human skeletal muscle from individuals with differing insulin sensitivities over a two hour time period. Of the 9 genes examined with this technique, 4 proved to be insulin responsive *in vivo* in insulin sensitive individuals. The proto-oncogenes c-Ha-ras, c-myc, and c-src all displayed a 2-4 fold transient increases in their respective RNA levels within 30 minutes of insulin infusion. In addition, myf-5, a muscle specific differentiation factor also proved to be insulin sensitive. The abundance of this RNA also increased 3-fold with a time course similar to those displayed by c-Ha-ras and c-src. The responses of c-Ha-ras and myf5 were diminished in insulin resistant individuals. C-myc, while responding to insulin stimulation in both groups showed overall lower levels in individuals with decreased insulin sensitivity. In contrast, c-src RNA levels increased in response to insulin in both groups. While RNA abundance of c-jun and insulin receptor were not altered over the two hour time course of insulin administration, the basal RNA levels were lower in individuals that are insulin resistant. This suggests that there are multiple insulin signal pathways that result in modifications in gene expression. Glut-3, Glut-4 and c-fos showed neither statistically significant increases in RNA levels nor were basal RNA levels altered by decreased insulin sensitivity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69044-02 PCCR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Resistance in Obesity and the Association with Membrane Phospholipid

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. Lillioja Visiting Scientist CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

.10

PROFESSIONAL:

.10

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

A convincing mechanism for the development of insulin resistance with the development of obesity has not yet been determined. A recent report has indicated that fasting plasma insulin concentrations, a measure of insulin resistance, is correlated with muscle phospholipid polyunsaturated fatty acid content. A similar relationship is also found with obesity. In a collaborative study with the authors of this work, we will examine the relationship of obesity, insulin resistance and membrane phospholipid content in Pima Indians to determine if obesity leads to insulin resistance by altering membrane phospholipid content.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69045-01 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of a Chromosome 4 Region Harboring a Gene Controlling Insulin Action

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. Prochazka	Senior Staff Fellow	CDNS, NIDDK
Others:	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	J. Tait	Research Fellow	CDNS, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Department of Laboratory Medicine, University of Washington, Seattle, WA.

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

.90

PROFESSIONAL:

.90

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Previous studies provided strong evidence for genetic factors controlling insulin action and insulin release in Pima Indians. Both, impaired insulin action (insulin resistance) in the skeletal muscle, and a defective glucose-stimulated insulin release appear to have a primary role in the development of NIDDM in this population. Initial analyses indicated a linkage of insulin action with the MNS blood group locus on the long arm of chromosome 4 (4q). We have expanded this observation by including several DNA-based polymorphic markers spanning approximately 133 map units (centiMorgans, cM) around MNS. The results were consistent with the presence of a putative gene on 4q controlling insulin action. Two markers separated only by 1 cM, the annexin V gene (ANX5) and the intestinal fatty acid binding protein gene (FABP2) showed the strongest linkage with the insulin resistance phenotype. Extensive typings of all currently known DNA markers in this region narrowed the chromosomal segment harboring the putative gene to about 5 cM encompassing ANX5 and FABP2. Because the only known functional genes in this region, ANX5 and FABP2, are unlikely candidates, we have started a long-range genomic cloning using the yeast artificial chromosome (YAC) approach to attempt the isolation of the putative gene. Our strategy includes isolation of overlapping YAC clones spanning the chromosomal segment of interest, and identification of novel highly polymorphic DNA markers which will be typed in individuals involved in this study. Clone(s) identified by markers showing the most significant linkage with the insulin resistance will be subject to the search for the candidate gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69046-01 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of Chromosome 19 in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. Prochazka	Senior Staff Fellow	CDNS, NIDDK
Others:	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

.55

PROFESSIONAL:

.55

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Non-insulin dependent diabetes mellitus (NIDDM) has a genetic component, and current evidence indicates that multiple genes contribute to the predisposition for this disease in Pima Indians. However, our knowledge about putative disease genes is very limited. Because of the genetic complexity of NIDDM, we have initiated a "candidate gene" approach to investigate genes coding for proteins which mediate insulin effects or play a role in glucose homeostasis. Our group have chosen chromosome 19 because it carries three potential candidate genes. The first is the gene for the insulin receptor (INSR). In view of the key role of this receptor in mediating insulin effects, and the abnormal responses of skeletal muscle in insulin resistant Pima Indians, INSR is an important candidate deserving a close attention. A second candidate on chromosome 19 is the gene for glycogen synthase (GS), selected because the activity of this enzyme is impaired in skeletal muscle of insulin resistant individuals, and the resistance is at least in part due to a decrease in insulin-mediated glycogen synthesis in skeletal muscle. Because Pima Indians have a high prevalence of obesity which is contributing to diabetogenesis, a gene for a hormone-sensitive lipoprotein lipase (LIPe) located also on chromosome 19 will be investigated. The enzyme encoded by LIPe is important for the utilization of fat as an energy source. Highly polymorphic DNA markers (either within, or in linkage with the candidate genes) are being analyzed in relation to overt diabetes, or to abnormal phenotypes (e.g. insulin resistance, obesity) which are contributing to NIDDM susceptibility.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69047-01 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mapping Chromosome 11 in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Thompson Senior Staff Fellow CDNS, NIDDK
Others: C. Bogardus Chief CDNS, NIDDK

COOPERATING UNITS (If any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

.26

PROFESSIONAL:

.26

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Chromosome 11 contains several candidate genes that may be involved in producing insulin resistance and type two diabetes. These candidate genes include the insulin gene, protein phosphatase 1 alpha, glycogen phosphorylase, c-Ha-ras, and IGF-2. In addition, a rare form of diabetes and one form of MODY (maturity onset diabetes in the young) diabetes have also been mapped to chromosome 11. In an effort to search for chromosomal regions and subsequently the genes involved in insulin resistance and type two diabetes polymorphic markers are being used to examine chromosome 11 at a 10 centimorgan resolution.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69048-01 PCCR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of Insulin Regulated Transcription Factors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Thompson Senior Staff Fellow CDNS, NIDDK

COOPERATING UNITS (If any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

.2

PROFESSIONAL:

.2

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous work on insulin regulation of gene activity revealed that insulin-resistance alters insulin regulated gene expression. Insulin-resistance alters immediate early gene expression in a gene specific manner, suggesting multiple insulin signal pathways that result in changes in gene expression. Two genes, c-Ha-ras and myf5, displayed the same types of insulin mediated responses in insulin-sensitive and -resistant individuals suggesting the same mode of regulation. To isolate the cis-acting sequences and the trans-acting factors that bind these sequences that are responsible for the changes in RNA levels for these two genes, genomic clones of myf5 have been isolated. The 5' regulatory region will be analyzed for insulin responsive elements. Once isolated the insulin responsive DNA element can be utilized to test insulin-sensitive and -resistant skeletal muscle for differences in the trans-acting factor that interacts with this element.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69050-01 PECR

PERIOD COVERED

October 1, 1991 to September 30, 1992

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Aspects of the Acute Insulin Response in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B. Thompson	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	R. Janssen	Summer Student	Arizona State University

COOPERATING UNITS (if any)

Indian Health Service; Arizona State University

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL STAFF YEARS:

.26

PROFESSIONAL:

.26

OTHER:

0.0

CHECK APPROPRIATE BOXES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous observations of the variation associated with the acute insulin response suggested that this phenotype was controlled by one or more genes. Glucokinase, which has been linked to the MODY (maturity onset diabetes of the young) form of diabetes, has been proposed as an intracellular glucose sensor, a likely candidate gene contributing to variations in glucose stimulated insulin responses. Similarly, GLUT2, the major glucose transporter of the pancreas could also be responsible for the variation observed in the acute insulin response. Polymorphisms at both of these genes have been examined for an association or linkage with the acute insulin response. Certain alleles of GLUT2 have been associated with the acute insulin response, and analysis of over 90 sibpairs suggests there is linkage between this region on chromosome 3 and the acute insulin response. Currently, a structural analysis of the coding regions for the gene encoding GLUT2 is underway.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 69049-01 PCCR
PERIOD COVERED October 1, 1991 to September 30, 1992		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) SSCP Analysis of PP-1 Alpha and Gamma in Relationship to Insulin Resistance		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: B. Thompson Senior Staff Fellow CDNS, NIDDK		
COOPERATING UNITS (if any) Indian Health Service		
LAB/BRANCH Phoenix Epidemiology and Clinical Research Branch		
SECTION Clinical Diabetes and Nutrition Section		
INSTITUTE AND LOCATION NIDDK, NIH, Phoenix, Arizona 85016		
TOTAL STAFF YEARS: .2	PROFESSIONAL: .2	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p> Previous studies on the serine/threonine protein phosphatase (PP-1) activity in skeletal muscle indicated increased amounts of activity in insulin-sensitive individuals, but increased protein amounts in insulin-resistant persons. Subsequently, studies on the steady state RNA levels of PP-1 alpha in human skeletal muscle revealed no differences between insulin-sensitive and resistant persons. In addition, a second isoform of PP-1 was cloned, suggesting that PP-1 activity may be a composite of several protein isoforms. Since the observed activity and protein differences are not accounted for by differences in the amount of RNA in these two groups, mutations in the coding regions of either of the two skeletal muscle PP-1 isoforms could explain the activity and proteins differences. Currently, the genes for both of the skeletal muscle protein phosphatase isoforms are being isolated. The genomic clones for both of these genes will be analyzed and suitable PCR primer pairs chosen from the non-coding regions that will allow amplification of PP-1 coding regions. The coding regions of both these genes will be examined in insulin-sensitive and -resistant persons for single stranded conformational polymorphisms (SSCP). SSCPs that result in amino acid changes will be further tested for a relationship to insulin resistance and type II diabetes. </p>		



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